# Regulation of Oxidative DNA Damage Repair by DNA Polymerase λ and MutYH by Cross-talk of Posttranslational Modifications

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**To my family**
**- Äiti, Isi, Tiia, Shiva, Little Foot and Paddington -**
**for all their love and endless support.**

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# Abbreviations

## In alphabetical order

| | |
|---|---|
| 8-oxo-G | 7,8-dihydro-8-oxoguanine |
| A | Adenine base |
| AFAP | Attenuated familial adenomatous polyposis |
| AP site | Apurinic/apyrimidinic site, abasic site |
| APC | Adenomatous polyposis coli protein |
| APE1 | Apurinic/apyrimidinic endonuclease 1 |
| *A.thaliana* | *Arabidopsis thaliana* |
| BER | Base-excision repair |
| C | Cytosine base |
| Cdk | Cyclin-dependent kinase |
| CS | Cockayne syndrom |
| DNA | Deoxyribonucleic acid |
| DSB | DNA double-strand breaks |
| *E.coli* | *Escherichia coli* |
| ES cells | Embryonic stem cells |
| FAP | Familial adenomatous polyposis |
| FEN1 | Flap endonuclease 1 |
| G | Guanine base |
| H | Histidine |
| $H_2O_2$ | Hydrogen peroxide, an oxidative agent |
| hmdUrd | 5-hydroxymethyluracil, a product of oxidative attack on the methyl group of a T base |
| HNPCC | Hereditary non-polyposis colon cancer |
| K | Lysine |
| MAP | MutYH associated polyposis |
| MEF | Mouse embryonic fibroblast |
| MMR | Mismatch repair |
| MMS | Methyl methanesulfonate, an alkylating agent |
| Mule | E3 ubiquitin ligase Mule |
| MutYH | MutYH DNA glycosylase |
| NER | Nucleotide-excision repair |
| NHEJ | Non-homologous end-joining |
| Ogg1 | Ogg1 DNA glycosylase |
| PCNA | Proliferating cell nuclear antigen |
| Pol | DNA Polymerase |
| PTM | Posttranslational modification |
| R | Arginine |
| ROS | Reactive oxygen species |
| RP-A | Replication Protein A |
| S | Serine |
| *S.cerevisiae* | *Saccharomyces cerevisiae* |
| SNP | Single-nucleotide polymorphism |
| SSB | DNA single-strand breaks |
| SSL | Synthethic sickness/lethality |
| T | Thymine base |
| T | Threonine |
| TCR | Transcription-coupled repair |
| TLS | Translesion synthesis |
| Y | Tyrosine |

# Summary

## English

It is crucial for a cell to maintain the genetic information encoded in DNA as stable as possible, in order to avoid deleterious diseases as cancer to establish themselves. Genomic stability relies heavily on components of various DNA repair machineries, which constantly guard the genetic integrity by correcting a multitude of mistakes and damages inflicted on the genome. Repair DNA Polymerases (Pols), such as Pols λ and β, are specialized in handling damaged DNA expertly, but they all exhibit considerably reduced fidelity when replicating long stretches of undamaged DNA. Thus, it is of pivotal importance that those Pols are meticulously regulated, as their misregulation could induce the establishment of mutations. 8-oxo-G is an abundant and highly miscoding oxidative DNA lesion arising from a variety of exogenous and endogenous sources. It has been shown that A:8-oxo-G mismatches, which are caused by incorrect bypass of 8-oxo-G by replicative Pols, can be accurately repaired *in vitro* and *in vivo* by a repair pathway that is coordinated by MutYH and Pol λ. Pol λ is phosphorylated by Cdk2/CyclinA in the late S and G2 phases of the cell cycle. This phosphorylation promotes stabilization of Pol λ by preventing it from being targeted for proteasomal degradation by ubiquitination. However, so far it has remained a mystery how this phosphorylation allows the stabilization of Pol λ mechanistically. Furthermore, it is unclear what other factors fine-tune and influence the control of the levels of Pol λ, and how the engagement of Pol λ in active repair complexes is coordinated. In the presented thesis, it is shown that the E3 ligase Mule mediates ubiquitination of Pol λ and thus targets it for degradation by the ubiquitin-proteasome system. Importantly, this control of Pol λ levels by Mule has functional consequences for the ability of mammalian cells to deal with 8-oxo-G lesions. Furthermore, it is demonstrated that the phosphorylation of Pol λ by Cdk2/CyclinA interferes with Mule-mediated degradation of Pol λ. Mechanistically, phosphorylation stabilizes Pol λ by increasing the affinity of phosphorylated Pol λ to its interaction partner, the chromatin-bound protein MutYH. This enhanced interaction promotes the recruitment of Pol λ to the chromatin. Since Mule is a protein found exclusively in the cytoplasm, it can not ubiquitinate the chromatin-bound fraction of Pol λ and thus not target it for proteasomal degradation. Consequently, MutYH appears to promote the stability of phosphorylated Pol λ by binding it to chromatin into active 8-oxo-G repair complexes, where it cannot be modified by Mule. On the other hand, the fraction of Pol λ that is not engaged in active repair complexes on chromatin is localized to the cytoplasm, where it is subject for proteasomal degradation by Mule-mediated ubiquitination. In summary, it is an intricate regulatory mechanism involving the cross-talk of two different PTMs - ubiquitination and phosphorylation - that allows the fine-tuning of the cellular Pol λ levels *in time and space*, and thus contributes to the maintenance of genetic stability.

## German

Es ist von äusserster Wichtigkeit für eine Zelle, dass die in der DNA kodierte genetische Information möglichst konstant gehalten wird, um die Etablierung verheerender Folgen von Mutationen zu vermeiden, welche zum Beispiel zur Krebsentstehung führen können. Die genetische Stabilität wird durch viele verschiedene DNA Reparaturmechanismen gewährleistet, welche durch ihre Korrekturfunktionen das Genom ständig gegen eine Vielzahl von schädlichen Einflüssen verteidigen. DNA Reparatur Polymerasen (Pols) wie Pol λ und β, die sich auf das Bearbeiten die Korrektur von DNA Schäden spezialisiert haben, sind hochpräzise in der Abfertigung von geschädigter oder veränderter DNA. Wenn sie jedoch lange Stränge unbeschädigter DNA replizieren, werden sie beträchtlich ungenauer und führen zur Synthese von fehlerhaften Basenpaarungen, welche wiederum Mutationen zur Folge haben können. Weil eine ungenaue Steuerung solcher Pols zu Mutationen führen kann, ist ihre präzise Regulation von höchster Wichtigkeit für die Erhaltung der genetischen Integrität. 8-oxo-G ist ein häufiger und stark fehlcodierender DNA Schaden, der durch die Oxidation der Base Guanin durch Agenzien aus verschiedensten endogenen und exogenen Quellen entsteht. Wenn replikative Pols über 8-oxo-G hinwegsynthetisieren, bauen sie häufig falsches Adenin statt des korrekten Cytosin ein, was zu Punktmutationen führen kann. Es ist gezeigt worden, dass solche A:8-oxo-G Fehlpaarungen *in vitro* und *in vivo* durch Zusammenarbeit von MutYH und Pol λ präzise korrigiert werden können. Pol λ wird durch Cdk2/CyclinA in der späten S und der G2 Phase des Zellzyklus phosphoryliert. Diese Phosphorylierung fördert die Stabilisation von Pol λ dadurch, dass Pol λ weniger ubiquitiniert und damit weniger durch den proteosomalen Degradationsweg abgebaut wird. Bisher ist es jedoch noch absolut unklar gewesen, wie genau diese Phosphorylierung die Stabilisation von Pol λ mechanistisch bewirkt. Zudem ist nicht bekannt, ob es Kofaktoren gibt, die eine Feinabstimmung dieser Regulation bewirken und somit zur Stabilisation von Pol λ beitragen. Zu guter letzt ist die Frage, wie die Rekrutierung von Pol λ in aktive Reparaturkomplexe vonstatten geht, noch gänzlich unbeantwortet. In der vorliegenden Dissertation wird gezeigt, dass Pol λ durch die E3 ubiquitin ligase Mule ubiquitiniert wird. Durch diese Ubiquitinierung wird Pol λ dem Abbau durch das Ubiquitin-Proteasom-System zugeführt. Diese Regulation des zellulären Gehalts an Pol λ hat funktionelle Folgen für die Fähigkeit von Säugetierzellen mit 8-oxo-G umzugehen. Des weiteren wird gezeigt, dass die Phosphorylierung von Pol λ durch Cdk2/CyclinA mit der Mule-mediierten degradation von Pol λ dadurch interferiert, dass sie die Affinität von Pol λ für ihren Bindungspartner MutYH steigert. MutYH ist ein chromatin-gebundenes Protein, was zur Folge hat, dass Pol λ so vermehrt an das Chromatin rekrutiert wird. Da Mule hingegen ein zytoplasmatisches Protein ist, kann es chromatin-gebundene Pol λ nicht abbauen. Daraus folgt, dass MutYH die Stabilisierung von Pol λ fördert, in dem es die phosphorylierte form von Pol λ vermehrt ans Chromatin bindet, wo sie in aktive Reparatur von 8-oxo-G Schäden miteinbezogen wird und nicht von Mule abgebaut werden kann. Die Fraktion von Pol λ hingegen, die nicht in aktive Reparaturkomplexe ans Chromatin gebunden ist, ist vermehrt im Zytoplasma lokalisiert, und wird deshalb durch Mule der proteasomalen Degradation zugeführt. Zusammenfassend wird gezeigt, dass die *zeitliche* und *räumliche* Feinregulierung der zellulären Pol λ Mengen durch eine ausgeklügelte Kommunikation der zwei unterschiedlichen posttranslationellen Modifikationen Ubiquitinierung und Phosphorylierung bewerkstelligt wird.

# Introduction

## General introduction

Deoxyribonucleic acid (DNA) is the structure that forms the basis of life, as it encodes the hereditary information that a cell needs in order to function. As the information that it encodes is absolutely vital, it is of pivotal importance that it is transmitted from one generation to the next in a very accurate fashion. The DNA is located in the nucleus of every living cell (with a very few exceptions like erythrocytes, which lose their cell nucleus during maturation). To guarantee survival and give rise to a subsequent generation, a cell has to be able to duplicate its DNA meticulously and also to repair eventual damage inflicted on it. The first task, the act of DNA duplication is termed 'replication' and is carried out by the so-called DNA polymerases (Pols) in cooperation with many other proteins. This procedure leads to the generation of two identical copies of the genetic material, which are subsequently distributed to the two newly formed daughter cells. DNA constitutes a chemically highly reactive molecule that is under constant attack by many exogenous and endogenous damaging agents. Thus, the second task - the repair of damages on DNA - is just as important as correct replication. Over billions of years of evolution, a multitude of strategies has evolved to cope with those two fundamental tasks in the most efficient and precise way possible. Only the most successful strategies have been conserved over this enormous amount of time by being meticulously relayed from one generation to the next. These strategies often consist of enormous protein machineries coordinating the complicated and fundamental events that have to be performed in order to enable a cell's survival and proliferation. Strikingly, the functions of these machineries, their single components and the mechanisms in which they work are often highly conserved in all the three kingdoms of life consisting of prokaryotes, archaea and eukaryotes (1).

The life of a cell occurs in a periodical and recurrent fashion in so called cell cycles. The cell cycle is divided in four phases. The G1 phase (gap) is the phase in which the cell's normal activity takes place, which is followed by the S phase (synthesis), the phase of DNA replication. After successful and complete replication of the entire genome, the cell enters the so-called G2 phase (gap 2), a short phase that enables the preparation and initiation of the actual cell division to create two daughter cells out of one single parental cell. Finally, the M phase (mitosis), which again can be subdivided into four different phases termed prophase, metaphase, anaphase and telophase, leads to the division of the parental cell into two identical daughter cells. A fifth cell cycle phase is termed G0 and represents a steady equivalent of the G1 phase for cells that have undergone full differentiation, have exited the replicative cell cycle and will not divide further under normal circumstances.

The organization of single cells into multicellular organisms, such as vertebrates and mammals, adds another layer of complexity to the intricate events occurring within single cells. In such conglomerates, an additional task that needs to be solved is the communication and coordination of each and every single cell to form a fully functional entity. In such an organisational masterpiece as the body is, the advantages for the entire unit are (and have to be) of higher interest than the fate of a single constituent or cell. In fact, an incredible amount of cells of a body are sacrificed to allow the rest to function at its best. For instance, during embryonic development, the formation of single fingers and toes is achieved by massive removal of the parts of tissue between the individual digits. This is accomplished by the execution of a programmed cell death termed apoptosis. Also cells that line the intestine or build the skin are only short-lived and are shed after very few days of life, a repetitive process that constantly occurrs during the entire lifespan of an individual. In view of such a huge, multifaceted and complicated symbiosis it becomes clear that the regulation of every single component is of pivotal importance. A loss of the ability of a single cell to act as part of the collective is mostly caused by mutations in DNA, either brought about by erroneous replication or a failure in the repair of DNA damage, as outlined above. Such mutations alter the genetic information and can lead to defective functions of the proteins encoded thereby. These alterations result in changes of a cell's behaviour and can lead to severe problems and disorders. Cells that the body has lost complete control over will start proliferating uncontrolledly and can ultimately culminate in the formation of a very well known and – sadly – predominant disease to humanity: cancer.

The distant hope to one day be able to cure cancer or even prevent it is a powerful source of motivation for thousands of people involved in cancer research. However, as Bruce Alberts, the editor-in-chief of the magazine 'Science', phrased it in a recent editorial: *"The remarkable advances in our knowledge of the chemistry of life achieved in the past few decades [...] could lead non-experts to assume that biologists are coming close to a real understanding of cells. On*

*the contrary, as scientists learn more and more, we have increasingly come to recognize how huge the challenge is that confronts us"* (2). In light of the immense complexity of even a single cell, which we are still far away from even grasping in all its abundance and intricacy, it will still take enormous struggle, effort, cooperation, innovation and perseverance to achieve the remote objective of conquering cancer.

The attempt of the work presented here is much more humble. It is merely to shed a tiny bit of light onto a struggle that is as old as life itself: a cell's endless endeavour to protect the integrity of its genetic information against the daily challenges that it is facing.

Nevertheless, as difficult and Daedalian the quest of understanding, challenging and ultimately hopefully defeating a disease as cunning as cancer might seem, to quote Theocritus, always remember:

*"While there is life there is hope, and only the dead have none".*

## Oxygen as a friend and enemy: the 8-oxo-G problem

Due to its highly reactive chemical nature, the integrity of cellular DNA is constantly challenged by a diversity of factors. As mentioned earlier, mistakes can arise during its replication by Pols (3), which can result in the generation of mutations. Furthermore, constant attack by exogenous and endogenous factors, which all can damage or alter bases, endanger the integrity of the information encoded by the DNA. Oxidative stress is one of those factors that damages DNA bases. It can arise from a variety of sources, such as UV light, cigarette smoke, ionizing radiation, inflammatory responses, cellular respiration and many more. The base guanine (G) is particularly susceptible to oxidative stress due to its low redox potential (4). This leads to the fact that many oxidised forms of G are present in the cell. 7,8-dihydro-8-oxoguanine (8-oxo-G) is recognized as one of the most important oxidative DNA lesions, because of its prevalence in DNA and its mutagenic potential in cells (5, 6). It has been estimated that the steady-state level of 8-oxo-G lesions is about $10^3$ per cell in normal tissues and up to $10^5$ lesions per cell in cancer tissues (5). The bulk of this damage is removed by the DNA repair systems, through the action of lesion-specific DNA glycosylases (such as OGG1, which removes 8-oxo-G opposite C and MutYH removing A opposite 8-oxo-G), which clean up the DNA from the modified bases (4). However, the lesions that go undetected by the repair systems, or those forming during the S-phase, might remain undetected and eventually pose a challenge to the replicative Pols encountering them. The prevailing view is that the replicative Pols α, δ and ε, are able to bypass an 8-oxo-G, but insert very frequently an adenine (A) instead of a cytosine (C) opposite the lesion (7). This leads to the formation an A:8-oxo-G mismatch, which can – if not recognised in time by MutYH – ultimately give rise to a CG → AT transversion mutation (7, 8).

The clinical relevance of CG → AT transversions has been shed light on in a study by Greenman et al. (9). The authors analysed over 1,000 somatic mutations corresponding to 518 protein kinase genes in 210 different human cancers. They found that in the 10 most common types of cancer that represent more than 50% of the overall cancer cases in humans, CG → AT transversions are often present. A large proportion of these CG → AT transversions might be attributed to 8-oxo-G lesions.

The miscoding potential of the 8-oxo-G lesion is dictated by the anti-syn equilibrium of the modified base, since, contrary to normal G, 8-oxo-G favours a syn conformation, enabling it to form a so-called Hoogsteen base pair with A (10). In the syn arrangement, the surface of the A:8-oxo-G mismatch resembles a normal Watson–Crick base pair in shape and geometry. On the contrary, when 8-oxo-G is present in the anti conformation to pair with C, it induces template distortions to avoid a steric clash between the C8 carbonyl oxygen and the sugar moiety (10). These template and enzyme distortions resemble, at the Pol level, those induced by mismatches (4). As a consequence, almost all human Pols studied so far show significant error-prone bypass of 8-oxo-G (10-17). Auxiliary proteins such as the processivity factor PCNA and single strand binding protein RP-A can influence the translesion synthesis ability of certain Pols. For example, the processivity factor PCNA has been found to influence bypass of 8-oxo-G lesions by Pol δ and to increase the efficiency of nucleotide incorporation both on undamaged templates and opposite a lesion by Pols η, ι and κ (reviewed in (18)). Importantly, it has been shown that the functional interaction of Pol λ with its auxiliary factors PCNA and RP-A ensures that C is incorporated opposite a template 8-oxo-G nearly 1200- fold more efficiently than A (7). Further work revealed that RP-A and PCNA coordinate the selection of Pol λ, while repressing the activity of Pol β in the repair of 8-oxo-G (19). Additional data by van Loon and Hübscher suggest the existence of a repair mechanism for 8-oxo-G coordinated by MutYH glycosylase and Pol λ (20). Taken together, these data indicate that human cells possess an accurate error-free bypass mechanism for 8-oxo-G involving Pol λ.

## DNA polymerases, the key enzymes for DNA replication and DNA repair

Ever since discovery of the DNA structure by Watson and Crick in 1953 it quickly became clear that there had to be specialized functional entities in the cell capable of reading and copying this genetic material, even though the authors themselves didn't exclude a spontaneous assembly of nucleotides as possible mechanism of DNA replication at first (21, 22). Shortly after that Meselson and Stahl demonstrated the existence of a so-called 'semiconservative mechanism of DNA replication' in the bacterium *Escherichia coli (E.coli)* (23). Subsequent work by Kornberg and his co-workers led to the discovery of a Pol that is capable of synthesizing DNA chains in a fashion that allows base pairing of A opposite thymine (T) and G opposite C, creating A:T and G:C base pairs (24, 25). This enzyme is now known as the bacterial Pol I.

Pols and their structures have been thoroughly reviewed in (3, 26). Universally, the structure of a Pol resembles a human right hand consisting of three domains, namely fingers, palm and thumb. When a Pol binds to DNA, conformational changes in the enzyme as well as the bound DNA lead to the formation of a so-called induced fit in the active site of the enzyme. Next a newly incoming nucleotide is attached in a phosphoryl transfer reaction to the 3'-OH group of the pre-existing DNA strand that is the 'primer' or the growing DNA strand. The repetition of this cycle will ultimately lead to the formation of DNA chains of megabases length.

So far, all known Pols have been grouped into the seven different families A, B, C, D, X, Y and RT based on sequence homologies. The following *Table 1* taken from (3) is intended to summarize and illustrate the seven Pol families and their members that are known to date. Prokaryotes (A, B, C and X), archaea (B, D, X and Y) and eukaryotes (A, B, X, Y and RT) all possess a different array of these families. The reason for the multitude of Pol families and Pols is the variety of functions and situations that they have to be capable of dealing with. For instance, the three Pols α, δ and ε are required during DNA replication. DNA repair, on the other hand, poses a much more complex task, as the bases that have to be bypassed, handled or extended are damaged and thus are very often miscoding or even blocking. For every kind of lesion or stress situation there is a specialized array of Pols prepared to perform what they are best at: trying to maintain the original sequence and thus protecting the genetic integrity.

***Table 1: An overview of DNA polymerase families and their members.***
Adapted from (3)

| Family | Viral | Bacteria | Archaea | Eukaryotes |
|---|---|---|---|---|
| **A** | T3 Pol | Pol I E. coli | | Pol γ |
| | T5 Pol | Pol I T. aquaticus | | Pol θ |
| | T4 Pol | | | Pol ν |
| | T7 Pol | | | |
| **B** | T6 Pol | Pol II E. coli | Pol BI | Pol α |
| | RB69 Pol | | Pol BII | Pol δ |
| | Adeno1 Pol | | Pol BIII | Pol ε |
| | HSV-12 Pol | | | Pol ζ/Rev3 |
| | Vaccinia Pol | | | |
| | Phi29 Pol | | | |
| **C** | | Pol III E. coli | | |
| | | Pol III B. subtilis | | |
| | | Dna E B. subtilis | | |
| | | Pol III T. aquaticus | | |
| **D** | | | Pol D | |
| **X** | ASFV3 Pol | Pol X D. radiodurans | Pol X M. mazei | Pol β (Pol IV S.c.4) |
| | | Pol X B.subtilis | Pol X M. thermauto trophicus | Pol λ (Pol LSP S.p5) |
| | | | | Pol μ |
| | | Pol X L. monocyto-genes | Pol X T. volcanium | Pol σ |
| | | Poļ X S. saprolyticus | Pol X F. acidarmanus | TdT6 |
| | | Poļ Ẋ S. aureus | | |
| | | Pol X D. reducens | | |
| | | Pol X A.aeolicus | | |
| | | Pol X T. thermophi-lus | | |
| | | Pol X T. denitrificans | | |
| | | Pol X T. aquaticus | | |
| **Y** | | Pol IV E.coli | Dpo4 Pol 7 | Pol η |
| | | Pol V E. coli | Dbh Pol 7 | Pol κ |
| | | | | Pol ι |
| | | | | Rev1 |
| **RT** | | RT8 | | Telomerase |

1: Adenovirus
2: Herpes simplex virus
3: African swine fever virus
4: Saccharomyces cerevisiae
5: Schizosaccaromyces pombe
6: Terminal deoxyribonucleotidyl transferase
7: From Sulfolobus solfataricus
8: Reverse transcriptases form retroviruses and lentiviruses

## DNA polymerase λ, an enzyme with many functions

Pol λ, together with Pols β, μ and TdT, is a member of the Pol X family. Those small, monomeric enzymes are relatively inaccurate when acting on longer stretches of undamaged DNA and are mainly implicated in the repair of small gaps in DNA spanning only a few nucleotides (27). While lower eukaryotes and plants possess only one member of the Pol X family, vertebrates have all four (reviewed in (3, 26)).

The POL L gene is localized on the human Chromosome 10 or the murine Chromosome 19 and gives rise to a 575 amino acid protein in humans and 573 amino acids in mice, with a molecular weight of about 67 – 70 kDa (28, 29). The structure of Pol λ consists of the typical palm, fingers, thumb and 8 kDa dRP lyase subdomains (amino acids 244 – 575). Furthermore, the N-term comprises the nuclear localisation sequence (NLS; amino acids 1 – 36), followed by a BRCT domain (amino acids 37 – 132) and a proline-serine-rich domain (amino acids 133 – 244) (*Figure 1*) (30). The sequence identity between the human C-terminal Pol β like core of Pol λ and Pol β is around 33%. Additionally to the normal template-dependent DNA polymerisation activity, Pol λ displays a multitude of catalytic activities, such as terminal deoxynucleotidyl transferase, dRP lyase and polynucleotide synthetase and it also efficiently synthesises DNA starting from an RNA primer (31, 32). Thus, Pol λ has been implicated to play a role in a variety of DNA repair processes, such as Base-excision repair (BER), Translesion synthesis (TLS) and Non-homologous end-joining (NHEJ) (33-38).

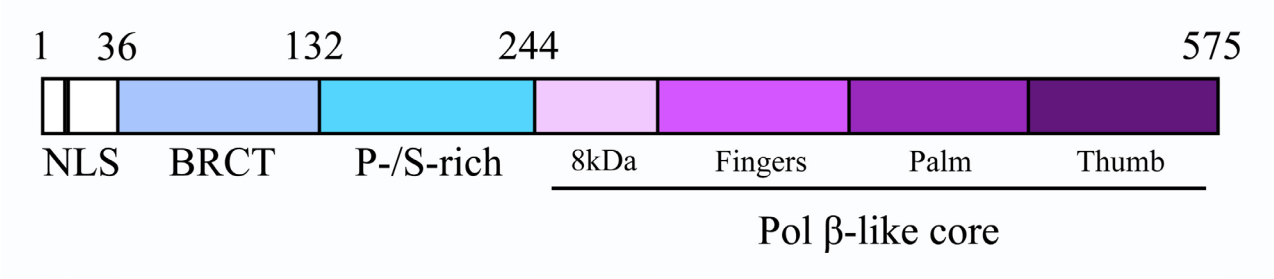


***Figure 1: Schematic presentation of the subdomains of DNA polymerase λ.***

When first identified independently by two groups in 2000, Pol λ was implicated to play a role mainly in DNA repair synthesis associated with meiosis. This was due to the fact that Northern blotting, in-situ hybridization analysis and immunostaining in mice showed Pol λ to be highly abundant in testis, although basal levels could be detected in all tissues that were examined (29, 39). Furthermore, the transcript levels of Pol λ were found to be highest during the period in which meiotic recombination occurs also in testes of adult mice, supporting the hypothesis of an involvement into meiotic DNA repair.

The first reports on Pol λ knockout mice were somewhat contradictory. A publication by Kobayashi *et al* stated, that Pol λ knockout (*Polλ*$^{-/-}$) mice display hydrocephalus, situs inversus, chronic sinusitis, and male infertility and implicated Pol λ to play a role in immotile cilia syndrome (40). Furthermore, mouse embryonic fibroblasts (MEFs) from those mice showed no sensitivity to X-rays, UV irradiation, $H_2O_2$ or MMS treatment. However, nowadays it is generally thought that the targeting approach used in this study did not only knock out Pol λ but also surrounding parts of DNA, where genomic analysis predicted the existence of a transcribed gene that is strongly conserved throughout evolution on the strand opposite of Pol λ. Thus the observed phenotype was most probably not only due to a lack of the original target Pol λ, but rather a side effect created by the loss of the adjacent DNA sequence, encoding for another important protein (41). The second group that generated Pol λ knockout mice, using a more targeted approach only deleting the catalytic domain of Pol λ, reported those mice to be viable and fertile (42). Additionally, an overall histological examination of various tissues from these mice failed to reveal any defect, thus contradicting the findings by Kobayashi *et al*. A report by Bertocci *et al* stated that Pol λ (together with its close relative Pol μ) seemed to be dispensable for immunoglobulin gene hypermutation, as assessed by analysis of heavy-chain sequences obtained from Peyer's patch derived B-cells. Follow-up work provided evidence that Pol λ is involved in V(D)J recombination, as Pol λ knockout mice displayed a shortening of the immunoglobulin heavy chain CDR3 by five base pairs on average (43). Also, the study showed that *Polλ*$^{-/-}$ MEFs did not display sensitivity towards X-rays, suggesting that Pol λ does not appear to contribute to DNA repair of DSBs. In contrast to that, a striking sensitivity of *Polλ*$^{-/-}$ primary MEFs towards oxidative stress - as induced by culturing them at 20% $O_2$ - was revealed. This sensitivity manifested itself in a reduced number of cells entering S phase and a subsequent entry in a growth-arrested state or premature cellular senescence, which was confirmed by the elevated expression of senescence-associated β-galactosidase. This senescence could be avoided by growing the cells at

3% $O_2$, suggesting an involvement of Pol λ in the protection against oxidative DNA damage.

Using a combination of *Pol*λ and *Pol*β single-knockout MEFs and neutralizing antibodies specific for both Pols, Braithwaite *et al* showed that Pol λ is capable of mediating a back-up BER activity when Pol β is not present. This was the first *in vivo* proof that Pol λ acts in a BER pathway (36). Follow-up work demonstrated that cells deficient in Pol λ display a hypersensitivity to oxidative stress induced by H2O2 or the incorporation of 5-hydroxymethyluracil (hmdUrd), whereas they behaved as wild-type cells when exposed to Methyl methanesulfonate (MMS) (35). Those findings provided further evidence that Pol λ could possibly be involved in the repair of specific DNA lesions *in vivo*. Along this line, the BER pathway for hmdUra involving Pol λ as well as interaction of Pol λ with the SMUG1 DNA glycosylase could be reconstituted *in vitro* by using purified proteins. Also, the requirement of the N-term of Pol λ for the recruitment of Pol λ to sites of laser-induced oxidative DNA damage together with SMUG1 was demonstrated. Finally, the authors claimed not to have able to observe recruitment of Pol λ to sites of DNA double-strand breaks (DSBs), in accordance with previous findings, though the data concerning this were not shown.

An involvement of Pol λ and a cooperation of Pol λ with Pol β in the repair of oxidative DNA damage in chicken DT40 cells was shown by Tano *et al* (44). While *Pol*β $^{-/-}$ or *Pol*λ $^{-/-}$ single knockout cells displayed only slight hypersensitivity to $H_2O_2$ treatment, a combined knockout strongly enhanced the sensitivity phenotype. *Pol*β$^{-/-}$ cells displayed a slightly elevated level of DNA single-strand breaks (SSBs), but the BER capacity of *Pol*λ $^{-/-}$ cells did not differ from wt cells. However, there was a clear increase in SSB from *Pol*β $^{-/-}$ to *Pol*β $^{-/-}$ *Pol*λ $^{-/-}$ double-deficient cells, suggesting that Pols β and λ can complement for one another in the protection against the genotoxic effects of $H_2O_2$ treatment. *In vitro* BER experiments fully corroborated the *in vivo* data and provided further proof that, in contrast to Pol β, Pol λ is not involved in the repair of damage mediated by alkylating agents.

In 2007, work from Maga *et al.* found that Pol λ, together with its co-factors Replication Protein A (RP-A) and Proliferating cell nuclear antigen (PCNA), is capable of inserting the correct C opposite 8-oxo-G 1200-fold more efficiently than the incorrect A *in vitro* (7). Experiments with whole cell extracts from wt and *Pol*λ$^{-/-}$ MEFs further underlined Pol λ's role in coping with 8-oxo-G *in vivo*. The idea of a role for Pol λ in 8-oxo-G repair was further strengthened when a study Maga *et al.* provided insight into the regulatory mechanism of polymerase-choice at an 8-oxo-G site (19). It could be shown that PCNA and RP-A act as molecular switches to allow the Pol λ-dependent highly efficient and faithful repair of A:8-oxo-G mismatches *in vitro* by using purified enzymes and *in vivo* by the use of *Pol*λ$^{-/-}$ and *Pol*β$^{-/-}$ knockout cell extracts. Furthermore, it could be demonstrated that the differential expression of Pols λ and β correlate with the development of a tumoural phenotype of immortalized human fibroblasts. Recently published data further demonstrate that MutYH and Pol λ are recruited specifically to sites of A:8-oxo-G mismatches but not to undamaged DNA in cell extracts. Immunofluorescence experiments indicated that those two proteins are involved in repair of oxidative DNA damage *in vivo* (20). In the same study, a fully reconstituted *in vitro* pathway involving MutYH, Apurinic/apyrimidinic endonuclease 1 (APE1), Pol λ, PCNA, RP-A, Flap endonuclease 1 (FEN1) and DNA ligase I was presented, suggesting the existence of a novel cellular response pathway to repair 8-oxo-G-associated damage.

In summary, Pol λ seems to be a multifunctional enzyme that has been ascribed a variety of roles. The feature of Pol λ that is momentarily in the main focus of our attention and also the main point investigated in the work presented here is its involvement in the repair of oxidative DNA damage. Probably this is one of the most difficult features of Pol λ to be assessed *in vivo* due to the numerous functional redundancies with a variety of other repair factors all working together immaculately in the cell to combat the mutational potential of oxidative DNA damage (thoroughly reviewed in our review shown below). The investigation and clarification of this role of Pol λ *in vivo* is likely to present still quite some surprises and confusions in the years to come.

## MutYH DNA glycosylase – the initiator of A:8-oxo-G repair

As discussed above, the problem of 8-oxo-G is that frequently a wrong A is incorporated opposite the lesion. This A:8-oxo-G mispair is the substrate for MutYH and it is the DNA glycosylase activity of MutYH that catalyses the excision of the wrongly incorporated A opposite an 8-oxo-G. More information about the catalytic mechanism and the role of MutYH in the repair of oxidative DNA damage can be found in an own review attached below (p. 18). In the following, the current knowledge about the role of MutYH *in vivo* is summarized.

### MutYH Associated Polyposis

MutYH dependent repair of 8-oxo-G was recognized to be important *in vivo* when Al-Tassan *et al* (45) found it to be mutated in the germline of a British family with three siblings showing clinical signs of Familial adenomatous polyposis (FAP). In FAP, a germline mutation in the Adenomatous polyposis coli protein (APC) gene gives rise to the formation of hundreds to thousands of adenomatous polyps in the colons of the affected patients. However, no such mutation in the APC gene could be found in the case of the affected British family. Further analysis of the patient material revealed an increased tendency of somatic CG → AT transversion mutations in the APC gene, a mutational pattern consistent with 8-oxo-G mediated mutagenesis. This observation led to the discovery of biallelic germline missense mutations in the MutYH gene in those patients. Subsequent work showed that the present point mutations in MutYH caused a decrease in MutYH's glycosylase activity for excision of A opposite 8-oxo-G, nicely correlating with the observed tumour phenotype (45-47). A follow-up study identified seven further unrelated patients with colorectal adenomas or carcinomas with a bias of CG → AT transversion mutations to be carriers of biallelic germline mutations for MutYH (48). This disorder that is inherited in an autosomal recessive mode, is now referred to as MutYH-associated polyposis (MAP). The prevalence of MAP is estimated to be around 1% of all colorectal cancer cases (49-56) and MutYH mutations have been found in 7% (57) and 10% (58) of FAP patients and 40% of Attenuated familial adenomatous polyposis (AFAP) patients, respectively (57).

Even though MAP is a disease discovered only very recently, many germline mutations in addition to the two found by Al-Tassan *et al* have been tracked down so far. The abundant body of literature investigating different single-nucleotide polymorphisms (SNPs) and their relevance to cancer development has been thoroughly reviewed (59, 60). Interestingly, no association of other genes than MutYH involved in BER or the repair of oxidative DNA damage with a multiple colorectal adenoma phenotype has been found (61).

Recently, MAP patients have been reported also to be more prone to extraintestinal tumours such as ovarian, bladder, skin and breast cancers (62). While one group reported an association of MAP with endometrial cancer (63), no association of MutYH genetic variants with endometrial cancer could be established by another (64). Biallelic MutYH germline mutations have also been found in one case to cause the Muir-Torré syndrome, a rare hereditary disease manifesting in gastrointestinal and cutaneous tumours (65). Another group reported a possible association of a MutYH biallelic mutation with endometrial cancer and Muir-Torré syndrome (66). In the case of two siblings homozygous for a MutYH frameshift mutation, the development of pilomatricomas, benign tumours derived from hair follicles, was observed during childhood before the onset of colorectal cancer (67). A substantial body of data has found more correlations of MutYH mutations with meningioma (68), both breast cancer and colorectal cancer (69), the risk of childhood acute lymphoblastic leukemia (70) and papillary thyroid cancer (71). Furthermore, six novel MutYH mutations in biallelic constellation and two novel monoallelic missense mutations causing FAP or AFAP were described (72) and a hitherto unknown 13-base-pair deletion on intron six of MutYH was found in two patients suffering from a relatively severe form of MAP.

### MutYH germline mutations and Mismatch Repair

An association of MutYH and Msh6 germline mutations in colorectal cancer patients has been described, suggesting that both genes act cooperatively and might thus confer an increase in the risk for colorectal cancer development when deficient simultaneously (73). Another earlier study however could not find an association between MutYH and Msh6 germline mutations when examining 64 Hereditary non-polyposis colon cancer (HNPCC) patients (74). Yet another finding suggested that the abrogation of both Msh6 DNA mismatch repair (MMR) and MutYH mediated base excision repair might be mutually exclusive in humans (75).

It has been reported that tumours from MAP patients show similarities to microsatellite instable cancers such as they are seen in cancers arising due to MMR deficiency (76). Microsatellite instability was also found in B-cell lymphoblastic lymphomas from *msh2*$^{-/-}$ *mutyh*$^{-/-}$ mice during tumour development (77). A study addressing the human leukocyte antigen I expression pattern on tumour cells derived from MAP patients showed that the its expression pattern is defective in 65% of the tested primary carcinomas, often occurring concomitantly with loss of the expression of beta-2-microglobulin (78). Those are features usually observed in MMR-defective tumours and are generally thought to represent immune evasion mechanisms beneficial to the cancer cells. Another study showed that MAP carcinomas often harbour chromosomal regions of copy-neutral loss-of heterogeneity and claim this is an important mechanism in the tumourigenesis of MAP (79).

## MutYH and age-related diseases

Recently, a Chinese study reported a mutation in the MutYH gene to be associated with increased levels of 8-OH-dG in the leukocytes of the carriers and increased interleukin-1 levels, suggesting impaired DNA repair and inflammation, possibly leading to the development of age-related or chronic diseases (80). Strikingly, the prevalence of healthy homozygous carriers decreased from 25.8% in the group of 20 – 29 years of age to 15.7% in the age group 50 – 59 years, hinting toward the fact that homozygosity for this mutation may have a strong influence on the development of age-related or chronic diseases or even mortality.

Decreased levels of MutYH have been implicated to be a susceptibility factor also in age-related retinal diseases (81) and the same group found increased mitochondrial DNA damage in rodent retinal pigment epithelium and choroid to coincide with the down-regulation of expression of MutYH among other DNA repair enzymes (82).

## MutYH -/- cells and mice

The data on cells and mice with biallelic deletion of MutYH are somewhat heterogeneous. Hirano *et al* reported MutYH knockout mouse embryonic stem cells (ES cells) to display a mutator phenotype, manifesting in a two-fold increased spontaneous mutation rate in a fluctuation assay, while the cells did not show any hypersensitivity towards $H_2O_2$ or menadione, a Vitamin K3 analogue that generates reactive oxygen species (ROS) by redox cycling (83). When Xie *et al* compared *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ MEFs to wt cells, they found the double knockout cells to be specifically sensitive to oxidants as $H_2O_2$ and T-Butyl-Hydroperoxide (an organic peroxide used for oxidation). Furthermore, those cells were found to react to oxidative stress with a reduction of S phase and accumulation of the cells in the G2/M phase, increased centrosome amplification and formation of multiple nuclei, suggesting also an involvement of MutYH and Ogg1 in the regulation of normal cell-cycle progression and cell division (84).

A study by Russo *et al* with *mutyh*$^{-/-}$ MEFs found that those cells accumulate 10 times more mutations than wild type cells at the *HPRT* gene, but they still displayed a rather mild mutator phenotype compared to *msh2*$^{-/-}$ MEFs. The combination of both knockouts to *mutyh*$^{-/-}$ *msh2*$^{-/-}$ lead to a mutation rate that did not differ significantly from the single *msh2*$^{-/-}$ MEFs, suggesting that both gene products are involved in the same pathway at least *in vitro* (77).

A study with *mutyh*$^{-/-}$ knockout mice by Xie *et al* revealed no significant difference in survival or tumour incidence between wild-type and *mutyh*$^{-/-}$ knockout mice after 14 months, leading to the conclusion that MutYH deficiency is not sufficient to cause a tumour-predisposition (85). This study also showed that when MutYH was knocked down simultaneously with Ogg1, the survival age for double knockout mice was significantly reduced and the tumour incidence was significantly increased compared to *mutyh*$^{-/-}$ or *ogg1*$^{-/-}$ single knockouts. The malignancies found in those double-knockouts were lung and ovarian tumours and lymphoma as well as some gastrointestinal adenoma and carcinoma. Interestingly, 75% of the lung tumours in these double-knockout mice showed an activating CG → AT transversion mutation at codon 12 of K-ras, a feature that is often detected in MAP tumours, but none could be found in the p53 gene or in the adjacent normal tissues. Additional heterozygosity for Msh2 (*mutyh*$^{-/-}$ *ogg1*$^{-/-}$ *msh2*$^{+/-}$) did not alter the total lung tumour incidence significantly but accelerated malignant lung and ovarian tumour formation in the *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ background. A complete knockout of Msh2 to generate triple knockout mice (*mutyh*$^{-/-}$ *ogg1*$^{-/-}$ *msh2*$^{-/-}$) led to an even higher tumuor incidence and decreased survival time but did not differ from the phenotype displayed by *msh2*$^{-/-}$ single knockouts. This might be due to the fact that the strong mutator phenotype of *msh2*$^{-/-}$ mice could mask any additional difference due to *mutyh*$^{-/-}$ and *ogg1*$^{-/-}$.

Analysis of spontaneous mutagenesis in the small intestine of *ogg1*$^{-/-}$ and *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ double deficient mice at the age of four to five weeks by using a prokaryotic rpsL transgene as a reporter revealed an increased mutational frequency in the double-knockouts but not in the *ogg1*$^{-/-}$ mice. Furthermore, the CG → AT mutation frequency was four-fold increased in *mutyh*$^{-/-}$ single knockouts, five-fold increased in *ogg1*$^{-/-}$ and a 41-fold increase could be observed in *mutyh*$^{-/-}$ *ogg1*$^{-/-}$, suggesting the existence of a cooperative function between Ogg1 and MutYH to prevent 8-oxoG-related mutagenesis in mammals (86). Another group also reported an additive effect in *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ on the age-dependent increase in 8-oxo-G levels specifically in liver, lung and small intestine compared to the single *mutyh*$^{-/-}$ knockouts, which showed only an age-dependent accumulation of 8-oxo-G in their livers comparable to *ogg1*$^{-/-}$ mice (87). Strikingly, the tissues that accumulated 8-oxo-G in *mutyh*$^{-/-}$ *ogg1*$^{-/-}$ mice were the same ones that showed increased cancer incidence in the study by Xie *et al* (85).

MutYH deficiency in a background of APC$^{min/+}$ mice has also been shown to enhance the occurrence of stop-codons in the APC gene by induction of CG → AT transversion mutations and thus to promote intestinal tumourigenesis, underlining the role of MutYH in prevention of CG → AT transversions and carcinogenesis *in vivo*, as it is also suggested to happen during pathogenesis of MAP (88).

In 2007 a study investigating a large cohort of mice kept for 18 months reported an increased susceptibility to spontaneous as well as stress-induced intestinal tumourigenesis in *mutyh*$^{-/-}$ mice, while their mortality was not increased in comparison to the wild type mice (89). These results strongly contradicted the data on *mutyh*$^{-/-}$ obtained by different groups thus far. Those *mutyh*$^{-/-}$ mice displayed five times more often intestinal tumours than wild type littermates, suggesting that presence of a MutYH deficiency is indeed sufficient to predispose mice to develop malignancies of the intestinal tract, such as lymphoma and adenoma. More impressively still, *mutyh*$^{-/-}$ mice displayed a dramatic increase in CG → AT transversion mutations and small intestinal tumours when treated orally for four weeks with $KBrO_3$, a known inducer of oxidative stress and particularly of 8-oxo-G. The authors claimed that the tumour-prone phenotype of the *mutyh*$^{-/-}$ mice might have been missed that far due to differences in the genetic background of the mouse strains and the older age at which the tumour burden was evaluated in their study. This is in line with the fact that many of the studies with *mutyh*$^{-/-}$ mice have been reporting a strong tendency towards age-dependent accumulation of 8-oxo-G in tissues.

As noted above, the combination of the two knockouts *mutyh*$^{-/-}$ and *msh2*$^{-/-}$ did not significantly alter the mutation rate compared to the single *msh2*$^{-/-}$ MEFs, suggesting that both gene products are involved in the same pathway at least *in vitro* (77). However, when the amount of oxidative DNA damage was examined in *mutyh*$^{-/-}$ *msh2*$^{-/-}$ double knockout mice, the loss of *mutyh*$^{-/-}$ contributed to a significant increase in several of the tested organs compared to *msh2*$^{-/-}$ single-knockout mice, suggesting an independent contribution of both genes to the maintenance of low levels of oxidative DNA damage within a mammalian organism. Surprisingly, both the development of metastasizing lymphoma and the time of death were significantly delayed in the *mutyh*$^{-/-}$ *msh2*$^{-/-}$ mice compared to *msh2*$^{-/-}$ single knockouts, suggesting that the cancer-prone phenotype of the double knockouts depends substantially on the activity of MutYH (77). The relationship of MutYH and MMR is reviewed in more detail in (90).

## Review:

# ‘Oxygen as friend and enemy: how to combat the mutational potential of 8-oxo-Guanine’

Barbara van Loon, Enni Markkanen and Ulrich Hübscher, *DNA repair*, 9 604-616, 2010

The following review, which I co-authored, is a thorough résumé of the current view of how a cell can cope with 8-oxo-G properly, thus avoiding the accumulation of severe DNA damage. An update on the literature since the review was published in 2010 is summarized below following the original publication.

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Mini-review

# Oxygen as a friend and enemy: How to combat the mutational potential of 8-oxo-guanine

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ABSTRACT

The maintenance of genetic stability is of crucial importance for any form of life. Prior to cell division in each mammalian cell, the process of DNA replication must faithfully duplicate the three billion bases with an absolute minimum of mistakes. Various environmental and endogenous agents, such as reactive oxygen species (ROS), can modify the structural properties of DNA bases and thus damage the DNA. Upon exposure of cells to oxidative stress, an often generated and highly mutagenic DNA damage is 7,8-dihydro-8-oxo-guanine (8-oxo-G). The estimated steady-state level of 8-oxo-G lesions is about $10^3$ per cell/per day in normal tissues and up to $10^5$ lesions per cell/per day in cancer tissues. The presence of 8-oxo-G on the replicating strand leads to frequent (10–75%) misincorporations of adenine opposite the lesion (formation of A:8-oxo-G mispairs), subsequently resulting in C:G to A:T transversion mutations. These mutations are among the most predominant somatic mutations in lung, breast, ovarian, gastric and colorectal cancers. Thus, in order to reduce the mutational burden of ROS, human cells have evolved base excision repair (BER) pathways ensuring (i) the correct and efficient repair of A:8-oxo-G mispairs and (ii) the removal of 8-oxo-G lesions from the genome. Very recently it was shown that MutY glycosylase homologue (MUTYH) and DNA polymerase λ play a crucial role in the accurate repair of A:8-oxo-G mispairs. Here we review the importance of accurate BER of 8-oxo-G damage and its regulation in prevention of cancer.


**Contents**



## 1. Functional consequences after oxidative stress

DNA is a dynamic structure, constantly subjected to changes. Some of these changes are alterations in the chemistry of normal bases, sugar moieties and the backbone of DNA, thus leading

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to DNA damage. Depending on the source, DNA can be damaged by (i) spontaneous reactions, mostly by hydrolysis; (ii) products of our metabolism, such as reactive oxygen and nitrogen species (ROS; NOS); and (iii) exogenous physical and chemical agents [1]. Exogenous sources of DNA damage include environmental agents such as ultraviolet (UV) light, ionizing radiation (IR), toxins, chemicals and pollutants [2–4]. Types of DNA damage that are produced include double-strand breaks (DSB), single-strand breaks (SSB), mismatches, inter- or intrastrand cross-links as well as chemical modifications of the bases or sugar moieties [5,6].

Besides deamination, spontaneous hydrolysis and nonenzymatic methylation, exposure to ROS is considered to be a major source of spontaneous DNA damage. ROS are constantly generated: in living organisms as byproducts of oxidative respiration in mitochondria, through redox-cycling events involving Fenton reactions mediated by heavy metals, as a consequence of IR, chemotherapeutic drugs and environmental exposure to transition metals and chemical oxidants [7–9]. In addition ROS are also produced by macrophages and neutrophils at the sites of infections and inflammations [10]. During oxidative metabolism in mitochondria, the majority of oxygen is converted to water ($H_2O$) and only 0.2–2% to ROS, due to leakage of the electrons directly to oxygen leading to formation of superoxide anions ($^{\bullet}O_2^-$) [11]. Superoxide anions can be further converted to hydrogen peroxide ($H_2O_2$), either spontaneously or through catalysis by superoxide dismutases. $H_2O_2$ can be next reduced to $H_2O$ or partially reduced to a very strong oxidant, the hydroxyl radical ($^{\bullet}OH$). Despite being less reactive than some other ROS, $H_2O_2$ has an important role in generation of oxidative damage and carcinogenesis due to its ability to easily diffuse through biological membranes. This enables $H_2O_2$ to reach fast other cellular compartments, such as nuclear DNA [12].

ROS damage vital cellular macromolecules such as proteins, lipids and DNA [13]. The first actors in the defense against the damaging effects of ROS are various antioxidants. Upon oxidative stress the ratio of oxidants to antioxidants increases. One of the most dramatic consequences of oxidative stress is the formation of DNA lesions, which might result in genomic instability and can lead to various diseases [6,14,15]. The cellular response to oxidative damage involves several processes, such as DNA repair, cell cycle arrest and apoptosis [16–18], while irreversible mutations contribute to oncogenesis [19].

### 1.1. 8-oxo-guanine is the best-studied oxidative DNA damage

DNA damage caused by ROS includes a large variety of lesions, such as base and sugar damages, DNA breaks and DNA-protein cross-links. There are more than 100 different types of oxidative base modifications identified in the mammalian genome and the most important ones are documented in Fig. 1 [20,21]. The low redox potential of guanine (G) makes this base especially vulnerable to oxidation [22]. Two main products of G oxidation are 7,8-dihydro-8-oxo-guanine (8-oxo-G) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (faPy-G). Eventually, 8-oxo-G can be further oxidized to produce guanidinohydantion and spiroiminodihydantion. Primary products formed from adenine (A) oxidation are 7,8-dihydro-8-oxo-adenine (8-oxo-A), 4,6-diamino-5-formamidopyrimidine (faPy-A) and ethenoadenine ($\varepsilon$A). Oxidation of pyrimidines leads mostly to the formation of thymine glycol, 5-hydroxycytosine and dihydrouracil (for more details see ref. [24]).

Among all the lesions created by ROS, 8-oxo-G is one of the most abundant and best characterized. 8-oxo-G arises by the introduction of an oxo group on the carbon at position 8 (C8) and a hydrogen atom to the nitrogen at the position 7 (N7). The estimated steady-state level of 8-oxo-G lesions is about $10^3$ per cell/per day in normal tissues and up to $10^5$ lesions per cell/per day in cancer tissues [23,24] (Table 1). The presence of 8-oxo-G is often used as a cellular biomarker to indicate the extent of oxidative stress [9]. 8-oxo-G in *syn* conformation is particularly mutagenic because of its strong ability to functionally mimic T. When present in the genome during DNA replication, 8-oxo-G represents a direct source of C:G to A:T transversion mutations (Fig. 3).

**Table 1**
Endogenous levels of different oxidative DNA damages per human cell per day.

| Type of oxidative damage | Number of DNA lesions[a,b] |
|---|---|
| 7,8-dihydro-8-oxo-guanine (8-oxo-G) | 1000–2000 |
| 2,6-diamino-4-hydroxy-5-formamidopyrimidine (faPy-G) | 1500–2500 |
| 7,8-dihydro-8-oxo-adenine (8-oxo-A) | 250–500 |
| 4,6-diamino-5-formamidopyrimidine (faPy-A) | 1000–2000 |
| Lipid peroxidation products (etheno-A, etheno-C) | 1000 |
| Ring saturated pyrimidines (thymine glycol) | 2000 |

[a] Data adopted from references [21,23,24].
[b] Estimates are for $3\times 10^9$ bp genomes.

### 1.2. Many cancers arise due to C:G to A:T transversion mutations

Somatic mutations include rearrangements and copy number alterations, small insertions and deletions, base substitutions, as well as epigenetic changes. These mutations occur in the genomes of all dividing cells, both normal and neoplastic. Driver mutations represent a class of somatic mutations that render clonal growth advantage and are often implicated in cancer development. These mutations are considered to be in 'cancer genes'. Mutations that do not contribute to the cancer development are called passenger mutations. So far there has been limiting information about the prevalence of somatic point mutations in human cancer genomes. Greenman et al. [25] analyzed somatic mutations in coding exons of 518 protein kinase genes in 210 diverse human cancers and reported different mutational signatures between different cancer types. C:G to A:T transversion mutations were identified to be among the most predominant somatic mutations in lung, breast, ovarian, gastric and colorectal cancers.

Cigarette smoke consists of a complex mixture of over 7000 compounds, including high levels of oxidants and ROS [26]. Among those compounds are more than 60 mutagens that can bind and chemically modify DNA [27]. It is suggested that cigarette smoke causes oxidative stress as well as a pro-inflammatory response in lung cells [28]. This can significantly contribute to the development of lung diseases, such as cancer. Lung cancer is a leading cause of cancer-related deaths world wide, with more than a million new patient cases identified each year [29]. Using massively parallel sequencing technology, 22,910 somatic substitutions were detected in a small-cell lung cancer cell line [30]. Among those C:G to A:T transversion mutations were observed in one third (34%) of all identified somatic substitutions. In addition, the first comprehensive catalogue of somatic mutations from an individual cancer genome identified C:G to A:T mutations as second most prevalent mutational signature in melanoma cells [31].

These very recent extensive studies of the patterns of somatic mutations in genomes of different cancer types shed light on the abundance of C:G to A:T transversion mutations and stress the importance of DNA repair systems that counteract accumulation of such events, thus preserving genome stability and integrity.

## 2. Base excision repair of 8-oxo-guanine

In order to repair DNA, cells have evolved different mechanisms. The base excision repair (BER) (Fig. 2) pathway is the primary and essential repair system involved in the removal of damaged DNA bases. The BER pathway is generally initiated by damage specific

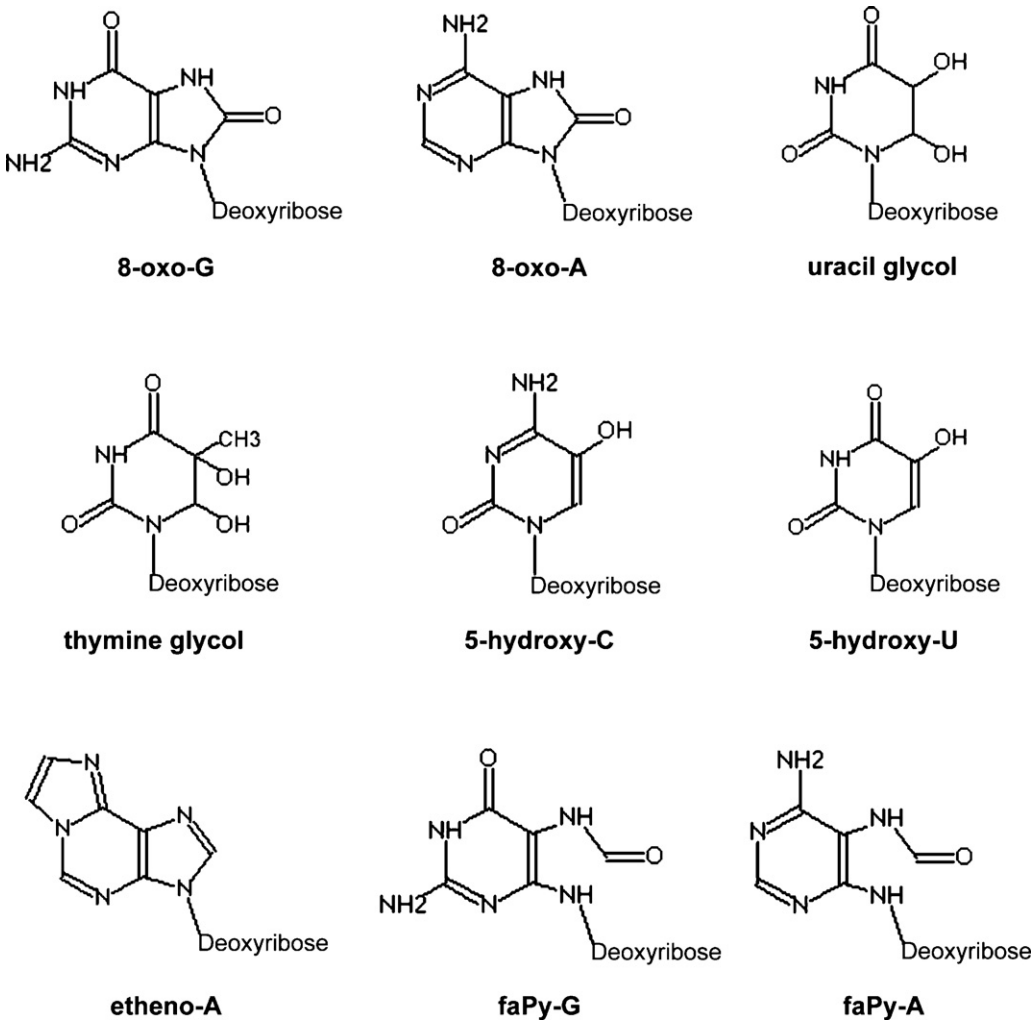


**Fig. 1.** The most common oxidative DNA lesions. 7,8-dihydro-8-oxo-guanine (8-oxo-G); 7,8-dihydro-8-oxo-adenine (8-oxo-A); uracil glycol; thymine glycol; 5-hydroxycytosine (5-hydroxy-C); 5-hydroxyuracil (5-hydroxy-U); ethenoadenine (etheno-A); 2,6-diamino-4-hydroxy-5-formamidopyrimidine (faPy-G) and 4,6-diamino-5-formamidopyrimidine (faPy-A).

DNA glycosylases that recognize nucleotide lesions and excise the damaged DNA bases by cleaving of the *N*-glycosylic bond between the 2′-deoxyribose and the damaged base [32–34]. All organisms possess several DNA glycosylases that can recognize and remove different DNA damages. The specificity of the BER pathway is determined by the type of the glycosylase that initiates it [32]. Based on their reaction mechanism DNA glycosylases are classified as mono- or bifunctional.

The monofunctional DNA glycosylases attack the anomeric carbon of the damaged base with an activated water molecule, thereby creating a free base and an apurinic/apyrimidinic (AP) site. The major 5′ AP endonuclease, apurinic/apyrimidinic endonuclease 1 (APE1) utilizes the AP site and generates a DNA repair intermediate that contains a single-strand (ss) break with 3′-hydroxyl and 5′-deoxyribose-5′-phosphate (5′dRp) termini [35]. The 5′dRp terminus is next excised by the dRp lyase activity of DNA polymerase (pol) β and a one nucleotide gap is created. The bifunctional DNA glycosylases, upon recognition and excision of the damaged base, incise by an associated AP lyase activity (β elimination activity) the strand 3′ of the AP site. The remaining unsaturated 3′ abasic fragment becomes a substrate for the APE1 and thus a one nucleotide gap is produced.

Further DNA repair is achieved through at least two distinct BER subpathways (Fig. 2): (i) short-patch BER (SP-BER) and (ii) long-patch BER (LP-BER). The feature distinguishing these two subpathways is the size of the repair patch synthesized by the repair DNA pol: (i) one nucleotide in the case of SP-BER [36], and (ii) two to 12 nucleotides in the case of LP-BER [37,38]. DNA pol β is the major repair polymerase in the SP-BER. In the LP-BER DNA pols with a capacitiy to perform strand displacement synthesis are required, a property that DNA pol β and the lagging strand replicase DNA pol δ possess. Moreover DNA pol β has been described to most likely incorporate the first nucleotide [39], while the subsequent elongation step is carried out by the replicative DNA pols δ or ε. Additional players in LP-BER are (i) replication factor C (RF-C) that is required to load proliferating cell nuclear antigen (PCNA) onto the DNA, (ii) PCNA, which is the sliding clamp for DNA pols and (iii) flap endonuclease 1 (FEN1), a structure specific nuclease that excises the displaced oligonucleotide [40]. The final ligation step in SP-BER is coordinated by DNA ligase III/X-ray repair cross complementing 1 protein (XRCC1) complex [41] and in LP-BER by DNA ligase I [42]. Recently an APE1-independent BER pathway has been described [43]. Upon base excision, the endonuclease VIII (Nei)-like proteins, NEIL1 and NEIL2, cleave DNA at the AP site by βδ elimination leaving at 3′ phosphate that is then removed by polynucleotide kinase (PNK).

To reduce the mutagenic effect of 8-oxo-G, many organisms have developed a three-component enzyme system (termed the 'GO system' after 8-oxo-G). In humans this system consists of the (i) 8-oxo-dGTPase (MTH1), and the two BER proteins (ii) 8-oxo-G DNA

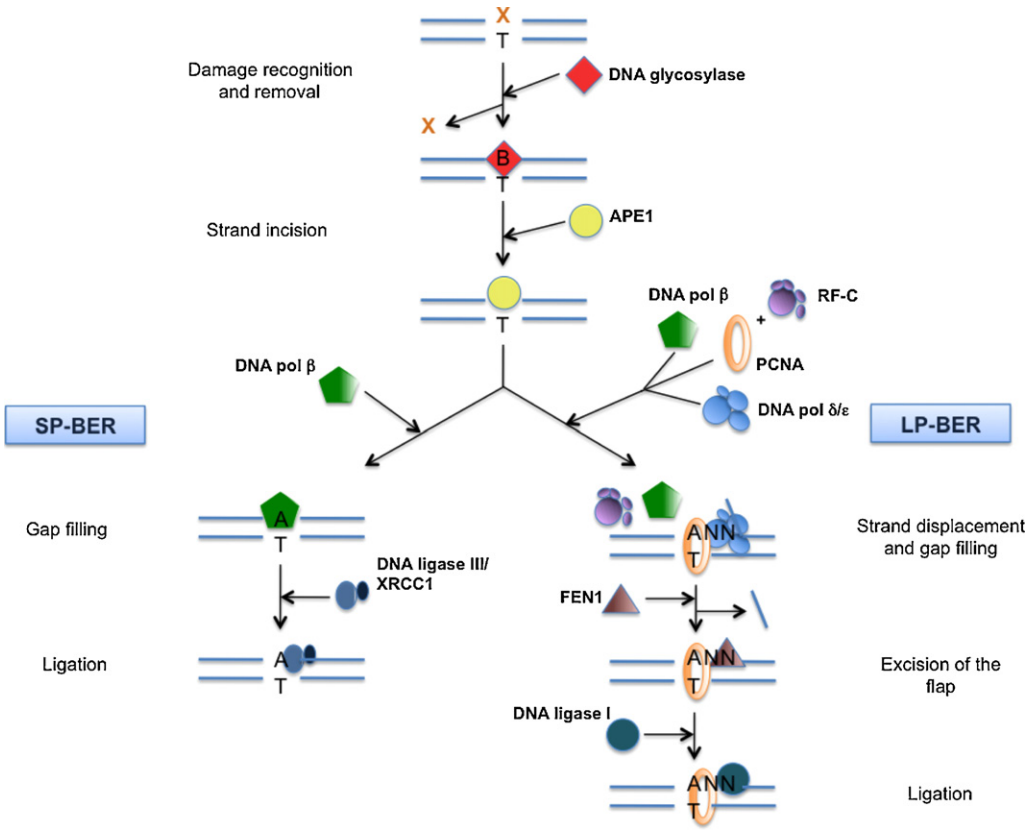


**Fig. 2.** Short-patch (SP) and long-patch (LP) base excision repair (BER) pathways. For further details, see text.

glycosylase (OGG1) and (iii) MutY glycosylase homologue (MUTYH) [44]. MTH1 hydrolyses 8-oxo-dGTP, thus removing it from the nucleotide pool so that it cannot be incorporated into DNA by DNA pols. OGG1 targets the C:8-oxo-G mispair, removes the lesion and in subsequent processing by other enzymes of the BER pathway the C:G base pair is restored. However, when the replicative DNA pols incoporate A opposite an 8-oxo-G, it is MUTYH that recognizes the resultant A:8-oxo-G base pair and removes the misincorporated A. The detailed mechanisms involving MTH1 have been reviewed recently [45], thus we will focus here entirely on the roles of the DNA glycosylases MUTYH and OGG1 in the repair of 8-oxo-G.

### 2.1. *MUTYH initiated base excision repair*

The *Escherichia coli* adenine DNA glycosylase, MutY is a member of helix-hairpin-helix (HhH) glycosylase family. MutY is an enzyme with mainly monofunctional DNA glycosylase and only weak and fully uncoupled AP lyase activities [46]. This glycosylase mediates removal of A paired with 8-oxo-G, G, faPyG, 5-hydroxyuracil or C.

Human MUTYH is encoded by the *MUTYH* gene, located on the short arm of chromosome 1 (1p32.1-p34.3). MUTYH is significantly larger than the bacterial protein and consists of catalytic core domain with an [4-Fe–4S] iron sulfur cluster in N-terminus [47,48], followed by an additional C-terminal MutT-like domain [49]. The extended N-terminal domain is involved in mitochondrial targeting of MUTYH and interaction with replication protein A (RP-A) while the C-terminal domain contains the nuclear localization sequence and the PCNA interacting motif [52–56]. There are at least two types of MUTYH protein: a mitochondrial and a nuclear form [52].

The full-length structure of MutY cross-linked to DNA containing an A:8-oxo-G mispair has helped to understand how the protein recognizes both A and 8-oxo-G [50,51]. The catalytic core and the MutT-like domains, both encircle the DNA, individually making close contacts to the appropriate DNA strand. The A is than flipped out into a deep active site pocket, which gets engaged in multiple direct hydrogen bonding and hydrophobic interactions [51], while 8-oxo-G stays in the base stack. The MutT-like domain establishes extensive contacts with 8-oxo-G, which in *anti* conformation gets stabilized in the MutY complex structure. Further, through acid-catalyzed protonation of the nucleobase, glycosidic bond cleavage is promoted. Interestingly, when mispaired opposite A, the 8-oxo-G in *syn* conformation is the energetically favored conformer.

The MUTYH activity can be modulated through interaction with other proteins. APE1 physically interacts with MUTYH [55] and enhances the MUTYH glycosylase activity [57]. Interestingly, this effect is independent of the APE1 endonuclease activity [57]. MUTYH also directly associates with both PCNA and RP-A [55]. This is of great importance since, in order to prevent mutations during DNA replication, the MUTYH activity must be directed to the newly synthesized strand. Indeed, it was shown that DNA replication stimulates the MUTYH initiated repair of A:8-oxo-G mispairs *in vivo* and interaction between MUTYH and PCNA is critical for this repair to occur [58]. In addition, MUTYH efficiently co-localizes with PCNA at replication foci [59].

### 2.2. *OGG1 initiated base excision repair*

The human OGG1 DNA glycosylase shares a significant homology with the *Saccharomyces cerevisiae* OGG1, but not with *E. coli* Fpg enzyme. All three enzymes are bifunctional DNA glycosylases with an associated AP lyase activity and ability to excise 8-oxo-G and faPyG when present in the DNA [60–69]. Finally, OGG1 can also remove 8-oxo-A [70,71].

Human OGG1 is present in at least four different splice forms [72]. The two most abundant ones have distinct cellular localizations. The α form of OGG1 is targeted in the nucleus and β form

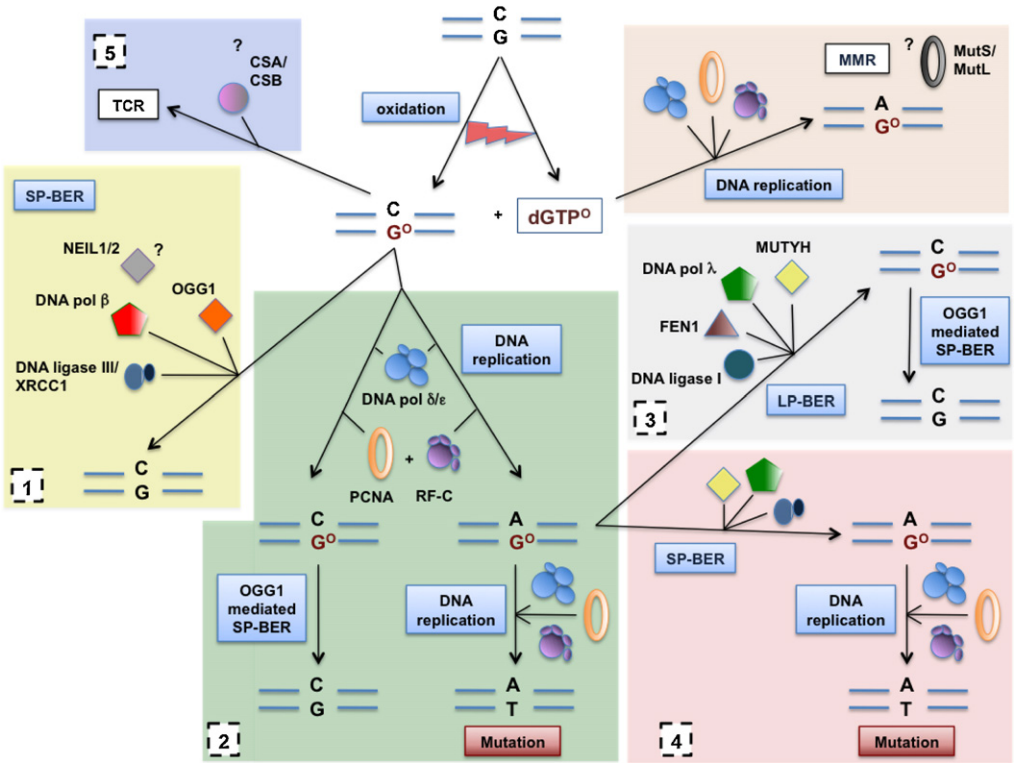


**Fig. 3.** Model of the different pathways to repair 8-oxo-guanine. Upon oxidation in the nucleus 8-oxo-G damage and 8-oxo-dGTP are produced among others (see Fig. 1). If 8-oxo-dGTP becomes utilized during DNA replication, it could be misincorporated opposite an A in the template strand (upper right). The resulting A:8-oxo-G mispair may further be processed and damage removed by MMR components, such as MutS and MulL. To repair this 8-oxo-G damage created in the DNA several pathways can be activated. (1) The majority of 8-oxo-G damages is removed by OGG1 mediated SP-BER. Upon the removal of the damage, DNA pol β is recruited and the final ligation step is mediated by DNA ligase III/XRCC1. NEIL1 or 2 could also play a role in this process. (2) When the damage is not removed before the DNA replication takes place, replicative DNA pols δ and ε with a high frequency inaccurately incorporate dAMP opposite 8-oxo-G. The resulting mispair in the next round of replication leads to C:G to A:T transversion mutations (right). If during DNA replication a correct dCMP gets incorporated opposite 8-oxo-G (left), in the subsequent step the damage will be removed by OGG1 mediated SP-BER as shown in (1). (3) In order to prevent C:G to A:T transversion mutations, A:8-oxo-G mispair is recognized by MUTYH in post-replicative LP-BER. Upon the removal of the inaccurate A by MUTYH and APE1, DNA pol λ in the presence of RP-A, PCNA and RF-C mediates accurate bypass by incorporating dCMP opposite 8-oxo-G and further elongating. The created flap is removed by FEN1 and DNA ligase I ligates a nick. In the subsequent OGG1 mediated SP-BER damage is removed. For simplicity RP-A, PCNA and RF-C are not shown next to DNA pol λ. (4) Upon the removal of A from the A:8-oxo-G mispair by MUTYH and APE1, repair synthesis can be mediated by a rather inaccurate DNA pol, or in minority of cases by DNA pol λ, resulting in formation of new A:8-oxo-G mispair. In subsequent SP-BER ligation of the nick is mediated by DNA ligase III/XRCC1. If this newly formed mispair becomes a substrate for DNA replication, C:G to A:T transversion mutations will occur. (5) When the transcription machinery encounters damaged DNA containing an A:8-oxo-G mispair TCR could possibly occur. CSA and CSB might play a role in this process. Note; XPC is not presented in this model, as there are not many indications at which step of 8-oxo-G repair this protein would be involved. For further details, see text.

in the mitochondria [56]. OGG1 contains two DNA binding motifs, a HhH and a $Cys_2$-$His_2$ zinc finger like motif. The general features used by OGG1 to recognize the lesion are well illustrated by the crystallographic X-ray analysis of the catalytically inactive mutant of OGG1 [73]. Upon DNA binding, the C:8-oxo-G base pair is disrupted and the 8-oxo-G flipped out of the double helix, while the enzyme undergoes local conformational changes induced by the preference for C in the opposite strand. Although OGG1 makes many specific connections with both nucleotides, only one hydrogen-bonding contact would not be possible with undamaged G. This is a bond between carbonyl oxygen of Gly42 of OGG1 and the hydrogen at position N7 of the 8-oxo-G. However, it is unlikely that the mechanism of the specificity for 8-oxo-G relays on a single hydrogen bond, so the mechanism for the discrimination is probably more complex. Upon excision of 8-oxo-G, OGG1 further utilizes the damaged base as a cofactor in the subsequent β-lyase cascade [74]. Several studies have indicated that OGG1 initiated repair is following the SP-BER pathway [75,76] (Fig. 3). In this process, DNA pol β seems to be responsible for the re-synthesis step. DNA pol β-deficient mouse cells exhibit a very low efficiency of C:8-oxo-G base repair, thus suggesting a direct involvement of SP-BER [75]. The *in vitro* reconstitution of BER of 8-oxo-G by using human enzymes, show that four proteins play a crucial role, namely OGG1, APE1, DNA pol β and DNA ligase I [77]. By addressing the subnuclear redistribution of the proteins initiating the repair of 8-oxo-G under oxidative stress conditions, recruitment of OGG1 to the open euchromatic regions is reported [78]. At the same time OGG1 is completely excluded from heterochromatin. This indicates that upon induction of oxidative damage DNA glycosylases are actively recruited to the regions of open chromatin.

Even though OGG1 initiated repair is the best characterized and the most important for recognition of C:8-oxo-G base pairs, several lines of the evidence indicate that this is not the only repair mechanism for the elimination of 8-oxo-G from genomic DNA [79,80]. Extracts from OGG1-deficient mouse embryonic fibroblast repair 8-oxo-G, although slowly and not very efficiently. Thus, beside the GO system, cells likely possess alternative repair pathways that can handle 8-oxo-G damage.

## 3. Additional DNA repair mechanisms involved in 8-oxo-guanine repair

Besides BER, other repair pathways are also important for the repair of oxidative DNA lesions. The nucleotide excision repair (NER) pathway recognizes helix-distorting base lesions, a broad category of damage that affects one of the two DNA strands [19,81,82]. A repair pathway closely linked to NER and possibly BER is transcription-coupled repair (TCR). This pathway targets lesions

**Table 2**
Proteins involved in the repair of 8-oxo-guanine.

| Protein | Function |
|---|---|
| OGG1 | DNA glycosylase, AP lyase, removes 8-oxo-G pared opposite C and formamidopyrimidines |
| MUTYH | DNA glycosylase, removes A paired opposite 8-oxo-G |
| NEIL1 | DNA glycosylase, AP lyase, removes 8-oxo-G paired opposite C |
| NEIL2 | DNA glycosylase, AP lyase, removes 8-oxo-G when present in bubble structures |
| APE1 | AP-endonuclease, 3′-phosphodiesterase |
| PNK | 3′-phosphatase |
| DNA polymerase β | DNA polymerase, dRP lyase |
| DNA polymeras λ | DNA polymerase, dRP lyase |
| DNA polymerases δ and ε | DNA polymerase, 3′ → 5′ exonuclease |
| PCNA | Auxiliary protein of DNA polymerases |
| RF-C | Loads PCNA AAA ATPase |
| RP-A | Auxiliary protein of DNA polymerases |
| DNA ligase III/XRCC1 | DNA ligase |
| FEN1 | Flap endonuclease. 5′ → 3′ exonuclease |
| DNA ligase I | DNA ligase |
| hMTH1 | 8-oxo-dGTPase |
| hMSH2 | Recognition of DNA mismatches |
| hMLH1 | Repair of DNA mismatches |
| CSB | Repair of 8-oxo-G from the overall genome |
| CSA | Repair of 8-oxo-G from the overall genome |
| XPC | Repair of 8-oxo-G from the overall genome |

that obstruct transcription [83,84]. In mismatch repair (MMR) small insertion/deletion loops and mismatched bases that result from replication errors or polymerase slippage are removed [85,86]. Additionally, some other DNA lesions are not repaired, but stay present in the genome and are bypassed during DNA replication by DNA pols that tolerate less stringent base pairing than replicative DNA pols [87].

Besides MUTYH and OGG1, the two DNA glycosylases NEIL1 and NEIL2 (Table 2) could possibly be involved in BER of 8-oxo-G (Fig. 3). Both NEIL1 and NEIL2 are bifunctional DNA glycosylases with a broad substrate range. Preferred substrates of NEIL1 are faPy-A, faPy-G, 5-hydroxyuracil or thymine glycol. NEIL1 can additionally excise an 8-oxo-G from a duplex DNA containing C:8-oxo-G base pair [88]. NEIL2 can also excise 8-oxo-G, but only when it is present inside of a bubble structure, while the ability of NEIL1 and OGG1 to excise 8-oxo-G from a bubble is almost completely lacking [89]. Since the bubble structure mimics a transcription intermediate, NEIL2 could be involved in the TCR. NEIL1 is specifically activated in the S-phase, suggesting that it could play a role in the removal of base lesions prior the replication [90]. NEIL1 can also stimulate OGG1, but these two DNA glycosylases do not stably interact [91]. OGG1 alone has a low turnover, but in the presence of NEIL1, similarly to APE1, its turnover is increased [91]. Both NEIL1 and NEIL2 interact with the BER proteins DNA pol β and DNA ligase III [43,92]. In addition NEIL2 is isolated from human cells in a complex with the BER proteins DNA pol β, DNA ligase III/XRCC1 and PNK, but not with APE1 [92].

The MMR system, coordinated by the hMutS and hMutL proteins, is the best-studied pathway for the correction of replication errors (Table 2). DNA glycosylases like MUTYH compete with the MMR machinery for the binding and processing of mispairs, such as A:8-oxo-G [57]. DNA glycosylases eliminate the DNA damage irrespectively whether it is located in the template or the nascent strand during DNA synthesis. Thus, if under these conditions a DNA glycosylase gets access to a mispair, the template strand will be corrected, thereby generating a mutation [93]. It has been suggested that the interaction of MUTYH with PCNA is crucial for the orientation of MUTYH to the newly synthesized DNA strand [55,59]. Discrimination between template and nascent strand established in this way would (i) allow MUTYH to excise A that gets incorporated opposite a template 8-oxo-G (G or C) only, (ii) while p MUTYH mutagenic processing of the parental A follow corporation of 8-oxo-dGTP (Fig. 3). Furthermore it has be that the human MMR proteins hMSH2 and hMSH6, com MutSα complex, bind to the A:8-oxo-G mispair and th interacts with MUTYH [94]. Binding of hMutSα to a subs taining an A:8-oxo-G mismatch is not affected by MUT other hand, the specific binding affinity of MUTYH to an mismatch is enhanced by hMutSα. Inactivation of the *l* leads to a mutator phenotype and increased cancer sus [95]. The 8-oxo-G levels in several organs of hMSH2 deficient animals are significantly higher than in the single knock-out animals, suggesting that *in vivo* MSH2 a provide separate repair functions and contribute indepe the control of oxidative DNA damage. Another MMR prote seems also to play a role in the 8-oxo-G repair, since cell in MMR, due to silencing of the *hMLH1* gene, have 4-f levels of 8-oxo-G than parental MMR-proficient cells [9

Published data suggest a possible role of both the syndrome B (CSB) and A (CSA) proteins in the repair c (Table 2). Both CSA and CSB are components of tra coupled nucleotide excision repair (TC-NER) and assist R dealing with transcription blocking lesions [19]. Human ing CSB are hypersensitive to γ-irradiation and defective i 8-oxo-G damage. Accordingly, 8-oxo-G accumulates mo deficient cells than in wild type cells after exposure to γ [97]. In human CSB-deficient cells OGG1 protein and r els are lower than in wild type cells [98]. In addition OGG1 co-localize upon γ-irradiation, but do not physic act. Depletion of the CSB protein from human whole ce leads to a reduction in the incision rate of 8-oxo-G [98]. Th suggests the possible effect of CSB in the catalytic proces G removal. Furthermore, CSB interacts with other BE such as APE1 [99]. Similarly to CSB, CSA is involved in th to oxidative stress. Human primary fibroblasts defecti show hypersensitivity to potassium bromate ($KBrO_3$), inducer of oxidative damage [100]. Even though seve show that CS proteins are involved in the repair of 8-o exact mechanism is still unknown. 8-oxo-G does not r strong transcription block and is efficiently bypassed in fashion by RNA pol II [101–103]. Recent measurements in gene expression upon presence of oxidized guanin bring some new insight in the possible interplay betwee OGG1 [104]. The absence of OGG1 does not have a stron the gene expression. In contrary lack of CSB activity le inactivation of the genes containing oxidized bases. Int this effect was attenuated by an additional OGG1 defici implicates that CSB could ensure the transcription funct process of DNA repair (Fig. 3) and accelerate the completi repair; while OGG1 would play a dual role in initiation of and gene inactivation [104].

Another member of the NER pathway, the Xeroderm tosum complementation group C (XPC) protein, migl involved in 8-oxo-G repair. Human primary skin cells de XPC patients are hypersensitive to oxidants and exhibi 8-oxo-G repair rate [105]. Complementation of these cel XPC wild-type gene product rescues the hypersensitivi as the defective DNA repair phenotype. Similarly, mice d XPC display an elevated sensitivity to oxidative damage, r lung carcinogenesis [106]. In addition, lympohocytes of t contain increased levels of *hprt* spontaneous mutations, lar C:G to A:T transversion mutations [107]. XPC has beer enhance the activity of OGG1, possibly through active dis of DNA glycosylase from its product, the AP site [105]. is abrogated in cells from patient with neurological symp have a single mutation in XPC [108]. The P334H mutatic

the interaction between XPC and OGG1, thus preventing the OGG1 stimulation.

## 4. Cells require a repair DNA polymerase that can accurately bypass 8-oxo-guanine

As mentioned above, the 8-oxo-G is efficiently, but inaccurately bypassed by the replicative DNA pols [109], resulting in the formation of a stable A(*anti*):8-oxo-G(*syn*) Hoogsteen mispair (Fig. 3). This mispair mimics a normal base pair and is not detected by the 3′ → 5′ exonuclease proofreading activity of the replicative DNA pols δ and ε. In order to reduce the mutational burden of ROS, the repair is initiated by MUTHY glycosylase that recognizes the A:8-oxo-G mispair and removes the A. During subsequent BER, cells need a specialized repair DNA pol that will catalyze with high preference the accurate bypass of 8-oxo-G.

*In vitro* studies have indicated that several DNA pols may be involved in BER [110–112]. DNA pol β, a member of DNA pol family X, was shown to be the major enzyme involved in gap filling [39,113,114], thus playing a central role in BER [115,116]. Another member of the DNA pol family X, DNA pol λ [117], has been implicated in BER [110,118], non-homologues end joining [119,120] and translesion synthesis [109,121,122]. In addition, earlier investigations suggested that the LP-BER pathway, involving aphidicolin-sensitive DNA pols, such as DNA pols δ and ε, is responsible for the repair of A:8-oxo-G mispairs [39,123]. However it is not known whether the aphidicolin sensitive DNA pols catalyze both insertion opposite 8-oxo-G and extension, or the extension only, since both DNA pol δ and ε are significantly inaccurate during 8-oxo-G bypass [109].

We have recently shown that DNA pol λ is very efficient in accurate bypass of 8-oxo-G lesion both on primed and one nucleotide gapped DNA templates [109,124,125]. On the other hand, human DNA pols α, δ and η show a much lower fidelity than DNA pol λ to incoporate the correct dCMP [109]. Moreover, the two auxiliary proteins PCNA and RP-A are able to additionally promote accurate gap filling by DNA pol λ. In the presence of RP-A and PCNA, DNA pol λ incorporates dCMP opposite 8-oxo-G on a one nucleotide gapped template 750-fold better than dAMP [124]. At the same time, these two auxiliary proteins prevent binding of DNA pol β to one nucleotide gapped 8-oxo-G template. Overall, the presence of RP-A and PCNA results in a 145-fold more efficient DNA pol λ than DNA pol β incorporation of dCMP opposite 8-oxo-G on one nucleotide gaps [124]. Immunofluorescence experiments in cells exposed to ROS suggest the involvement of MUTYH and DNA pol λ in the 8-oxo-G repair [125]. Additionally, upon treatment of the cells with $H_2O_2$, a dramatic increase in protein levels of MUTYH and DNA pol λ is observed, directly indicating the activation of MUTYH/DNA pol λ-dependent repair pathway [125]. A cross-linking assay with human whole cell extracts provides evidence that MUTYH, DNA pol λ, PCNA, FEN1 and DNA ligases I and III are the crucial components of A:8-oxo-G repair pathway [125]. *In vitro* reconstruction experiments further show that the accurate repair of A:8-oxo-G mispairs involves MUTYH, DNA pol λ, FEN1 and DNA ligase I [124] (Fig. 3). However, it is suggested that when DNA pol λ, or one of the other repair DNA pols, incorporates dAMP opposite 8-oxo-G, an inaccurate loop is initiated. The ligation step in this loop is mediated by DNA ligase III/XRCC1, resulting in the formation of the product that contains an A:8-oxo-G mispair [125] (Fig. 3). The product of this step could possibly be further recognized by MUTYH and APE1, thereby creating another chance for the accurate repair to occur.

The X-ray crystal structure of the catalytic domain of human DNA pol λ helps in understanding why exactly DNA pol λ, and not any other of the DNA pols from X family, has such a remarkable preference for accurate bypass of 8-oxo-G. A structure of human DNA pol λ in complex with primer/template containing a two nucleotide gap shows that DNA pol λ has two DNA binding sites that bend DNA, thereby exposing the 3′ primer terminus [126]. The polymerase domain binds the primer terminal base pair and the upstream duplex, while the 8 kDa domain binds the DNA downstream of the gap. The interactions between the polymerase domain and the duplex DNA upstream of the primer 3′ terminus are not extensive [126]. Furthermore, the template-binding groove in DNA pol λ is not as positively charged as in DNA pol β, suggesting weaker interactions with the template strand [127]. This feature could be crucial for the accurate bypass of 8-oxo-G damage. However, the binding of the DNA downstream of the gap is predominantly mediated via the interaction of 5′ terminus of the gap with the positively charged residues of the 8 kDa domain. This binding is further enhanced by the presence of 5′ terminal phosphate, which at the same time stimulates the DNA pol λ polymerase activity on a gapped substrate [127]. A very recent crystal structure reveals that DNA pol λ, upon binding to 2nt gapped double-stranded DNA, "scrunches" the template strand and binds the additional uncopied template base in an extrahelical position within a highly conserved binding pocket [128]. Mutation of the amino acids within this pocket into alanine results in less processive gap filling and less efficient DNA repair [128]. Thus, scrunching of the template strand by DNA pol λ likely occurs during gap filling DNA synthesis and is of advantage to the repair of 8-oxo-G.

Though the present data strongly suggests the role of DNA pol λ in BER and the repair of 8-oxo-G, many questions still stay unanswered. Among all, it remains to be explored how the MUTYH and DNA pol λ coordinated repair of A:8-oxo-G mispairs is achieved on the chromatin and how is it regulated.

## 5. Regulations of the key proteins involved in the repair of 8-oxo-guanine

The importance of the proper function of the BER pathway has become evident from numerous clinical studies. Mutations in BER proteins observed in various human pathologies provide extremely interesting and informative data and contribute to better understanding of the role of those proteins *in vivo*. The components of DNA damage repair have to be tightly regulated in order to ensure their proper and timely function. In addition to mutations that directly affect the protein activity, mere dysregulation of some of the components of the DNA repair machinery might well contribute to the development of human diseases [129]. Since the relevance of mutations in components of BER pathway in human pathologies has been reviewed recently [6,14], we would like to focus in this last chapter on what is known about the regulation of MUTYH, OGG1 and DNA pol λ more in detail.

### 5.1. Regulation of MUTYH

Data alluding to how MUTYH is regulated *in vivo* exist, but no detailed mechanism has been established yet. Findings from Boldogh et al. [59] show that nuclear MUTYH co-localizes with BrdU and PCNA at replication foci. Furthermore, nuclear levels of MUTYH increase 3- to 4-fold during progression through the cell cycle and to reach a maximum in S phase compared to early G1 phase. These data are in line with findings of Hayashi et al. [58] who, by investigating A:8-oxo-G repair in an *in vivo* repair system, observed a 14-fold higher repair efficiency when a replication-proficient substrate was used compared to a not replicating one. In addition, the replication-associated repair of A:8-oxo-G by MUTYH is dependent on its interaction with PCNA. These findings are in clear accordance with a replication-associated activity of MUTYH.

**Table 3**
Post-translational modifications of key proteins involved in BER of 8-oxo-guanine.

| Protein | Post-translational modification |
|---|---|
| OGG1 | Phosphorylation [149,150], acetylation [152] |
| MUTYH | Phosphorylation [131] |
| APE1 | Phosphorylation [153–155], acetylation [156], ubiquitination [157] |
| DNA polymerase λ | Phosphorylation [134,135], ubiquitination [135] |
| DNA polymerase β | Phosphorylation [158,159], acetylation [160], methylation [161], ubiquitination [136,137] |
| DNA polymerase δ | Phosphorylation [162,163], SUMOylation [164], ubiquitination [164] |
| PCNA | Phosphorylation [165], acetylation [166], SUMOylation [133,167], ubiquitination [133,167] |
| RF-C | Phosphorylation [168,169], ubiquitination [170] |
| RP-A | Phosphorylation [171–174] |
| XRCC1 | Phosphorylation [175–177], SUMOylation [178], ubiquitination [179] |
| DNA ligase III | Phosphorylation [180] |
| FEN1 | Phosphorylation [181,182], acetylation [183–185] |
| DNA ligase I | Phosphorylation [186–188] |

Very little is known about post-translational modifications of MUTYH. Parker et al. [130] investigated the role of MUTYH in a cell line displaying a C:G to A:T transversion mutator phenotype. Interestingly, the observed phenotype was not caused by a mutation in the MUTYH gene, but rather by a decrease in MUTYH mRNA and protein levels. Subsequent work by the same group showed that MUTYH is phosphorylated by protein kinase C (PKC) *in vitro* and that this phosphorylation enhances MUTYH A:8-oxo-G repair activity *in vitro* [131] (Table 3). Accordingly, treatment with the PKC activator phorbol-12-myristate-13-acetate (PMA) leads to phosphorylation of MUTYH and to an increase in A:8-oxo-G repair activity. This increase is abolished upon immunodepletion of MUTYH from the cell extracts and leads to a decrease in the elevated 8-oxo-G levels. Even though phosphorylation of MUTYH by other kinases can not be excluded, these findings implicate that phosphorylation of MUTYH is a prerequisite for its stabilization and activity and it remains to be investigated in further detail. In addition, the findings by Parker et al. support the idea that dysregulation of components of BER might contribute to the generation of mutations in a similar extent as a germline mutation in one of the genes encoding the BER proteins does.

MUTYH and the Rad9-Rad1-Hus1 complex (9-1-1 complex) interact *in vitro* and this interaction stimulates MUTYH activity *in vitro* [132]. In addition Rad9 and MUTYH co-localize in HeLa cells upon treatment with $H_2O_2$, suggesting that either MUTYH serves as a sensor for DNA damage, subsequently recruiting the 9-1-1 complex to the damage, or that the 9-1-1 complex is loaded independently of MUTYH onto damaged DNA, merely stimulating its activity. Further studies are needed in order to shed more light on the function, regulation and consequences of the interaction of these two proteins.

Taken together, it would be extremely interesting to investigate the cell-cycle phase dependent association of PCNA with MUTYH more closely, thus possibly gaining better insight into the regulatory mechanisms of this interaction. Post-translational modifications, such as ubiquitination or SUMOylation of PCNA at Lysine 164 (reviewed in [133]) or phosphorylation of MUTYH represent an intriguing possibility for such a subtle regulation.

### 5.2. Regulation of DNA polymerase λ

The first data about regulation of DNA pol λ *in vivo* came from a study performed in our laboratory by Frouin et al. [134], showing that the cyclin-dependent kinase 2 (Cdk2)-cyclin A complex interacts with and phosphorylates DNA pol λ *in vitro* in the prolin-serine rich domain (Table 3). This phosphorylation does not affect DNA pol λ polymerization activity and is decreased when PCNA interacts with DNA pol λ. Finally, phosphorylation of DNA pol λ *in vivo* during the cell cycle mimics the phosphorylation pattern of Cdk2-cyclin A. In a follow-up study, Wimmer et al. [135] could demonstrate that DNA pol λ is phosphorylated at Threonine 553 and that this phosphorylation protects the enzyme from being ubiquitinated (Table 3) and subsequently degraded via the proteasomal pathway. This phosphorylation and subsequent stabilization takes place in the late S and G2 phase, consistent with a possible role of DNA pol λ in the post-replicative repair of A:8-oxo-G mispairs. It remains to be investigated how and to what extent DNA pol λ is regulated *in vivo*. Post-translational modifications such as ubiquitination, as has been shown for DNA pol β [136,137], represent one possible means of regulation, and the role of phosphorylation in the stability and subcellular localization of DNA pol λ remains to be elucidated in more detail.

### 5.3. Regulation of OGG1

The regulation of OGG1 has been investigated by various approaches, but the complete mechanism is not completely understood yet. When investigating the OGG1 promoter, Dhénaut et al. [138] found a lack of TATA or CAAT boxes, suggesting that OGG1 is a housekeeping gene. In line with this finding is also the notion that OGG1 expression does not vary during the cell cycle. Lee et al. [139] show an up-regulation of OGG1 expression in HCT116 cells treated with MMS through the induction of the transcription factor nuclear factor YA (NF-YA). This increase further correlates with an increase in enzyme activity, providing to the cell functional protection from MMS. Interestingly, OGG1 mRNA or protein levels do not change upon the treatment of HCT116 cells by $H_2O_2$ [138]. Induction of OGG1 by oxidative stress seems to be tissue- and cell-type dependent, as there are several reports showing an induction (e.g. [140,141]), while others find no change in OGG1 levels upon treatment with oxidative agents (e.g. [138]). Habib [142] defined a novel mechanism of regulation of OGG1 through Tuberin in HEK and MEF cells. Knockdown of Tuberin in those cells leads to a downregulation of OGG1 and accumulation of 8-oxo-G, with NF-YA acting as important regulator of OGG1 transcription.

The activity of the repair enzyme OGG1 in the nucleus is upregulated significantly by regular exercise in rats [143] and activation of OGG1 and other BER components by ischemia may contribute to neuroprotection by enhancing the repair of endogenous oxidative DNA damage after ischemic injury [144,145]. Observation of age-dependent down-regulation of OGG1 in accelerated senescence mice hints towards an involvement of OGG1 activity in age-dependent diseases [146]. Mirbahai et al. showed that induction of OGG1 activity upon $KBrO_3$ treatment is not a result of induction of OGG1 gene expression, but rather protein stabilization [147]. Taken together, there seem to be multiple layers and mechanisms of induction of OGG1 expression that are either cell-type or damage specific.

Amouroux et al. [78] revealed that induction of 8-oxo-G leads to recruitment of OGG1 to euchromatin regions rich in RNA and

RNA pol II while being completely excluded from heterochromatin. This recruitment does not require direct interaction of the protein with the oxidized base. Results by de Souza-Pinto et al. [148] indicate that RAD52 interacts with OGG1 *in vitro* and *in vivo*, cooperates with OGG1 to repair oxidative DNA damage and enhances the cellular resistance to oxidative stress. OGG1 is shown to be preferentially associated with chromatin and the nuclear matrix during interphase and to become associated with chromatin during mitosis. Recently, OGG1 has been suggested to be a microtubule-associated protein during interphase and mitosis. In this way, by "riding" on microtubules, the redistribution of OGG1 within the cell might be facilitated [149]. Finally, there is one report concerning the degradation of OGG1, showing that upon oxidative stress and cisplatin exposure, OGG1 is degraded in a Calpain-dependent manner [150].

OGG1 was shown to be phosphorylated (Table 3) on a serine residue *in vivo* possibly by PKC [149]. Additionally, the kinases cyclin-dependent kinase 4 (Cdk4) and cellular Abelson murine leukemia viral oncogene homolog 1 (c-Abl) phosphorylate and interact with OGG1 *in vivo*. The phosphorylation of OGG1 by Cdk4 stimulates its 8-oxo-G incision activity *in vitro* [151]. OGG1 was also shown to be acetylated by p300 (Table 3). This acetylation predominantly takes place at the residues K338 and K341 and results in an enhancement of OGG1 activity *in vitro* and *in vivo* [152]. Thus, there is extensive data underlining OGG1 role in 8-oxo-G repair *in vivo*, but many questions remain to be elucidated. How, when and where the expression of OGG1 is regulated, how it is recruited to the chromatin parts that are in need of repair and what determines its stability are just some of the matters that remain to be investigated in the future.

## 6. Conclusions and perspectives

Oxidation damage by ROS is a frequent event in any living organism. High levels of ROS can lead to mutations, possibly result in transformation and eventually give rise to cancer. We have summarized the actors that might be important to keep the steady-state level of oxidative damage low enough not to harm an organism. Basic ideas and mechanisms about the repair possibilities are known, but we are still at an early stage of understanding the complete pathways in detail. As can be seen from Table 3 and the references therein, most components of the BER pathway are shown to be post-translationally modified by phopshorylation, acetylation, methylation, ubiquitination and SUMOylation [153]. Upon genotoxic stress, these modifications give numerous possibilities to regulate the repair actors in time and space. Studies of recruitment of modified proteins to chromatin during the cell cycle and under various stress conditions should further illuminate the repair processes.

In conclusion, a more thorough understanding of the exact mechanisms that help cells to cope with oxidative DNA damage, in particular 8-oxo-G, is needed. This should lead to new insights and understanding as to what is going wrong when malignancies arise and, more important, how to combat them.

## Conflict of interest

The authors declare no conflict of interest.

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## Recent developments in the field of 8-oxo-G repair – an update since 2010

### Repair of 8-oxo-G lesions

If the DNA was simply an elongated loose molecule floating freely in the cell, repair of DNA lesions would be easily feasible anywhere anytime. However, within a cell's nucleus, DNA is never present as a linear molecule, but highly organized into a complex structure called chromatin, consisting of DNA, histones and many other proteins forming complexes that guarantee the correct spatial and structural arrangement of this huge, compact molecule. Due to the complexity of this structure, it seems logical that the accessibility for repair complexes of oxidative DNA damage can be restricted by different chromatin configurations, as was demonstrated in a study by Khoronenkova *et al* (91). They provide evidence that chromatin remodeling mediated by the deubiquitinating enzyme USP7 is important for BER of oxidative lesions, as transient USP7 knockdown did not change the levels or activity of BER enzymes, but significantly reduced chromatin DNA accessibility and consequently the rate of repair of oxidative lesions.

Not only the chromatin status but also the differentiation state of a cell seems to play a role in determining its capacity to perform oxidative DNA damage repair, as shown in a study using undifferentiated ES cells or ES cells differentiated for 0, 4 and 7 days (92). Amounts of 8-oxo-G and the expression levels of Ogg1, MutYH and MTH1 in the cells was determined after treatment with $H_2O_2$ and it was found that, while levels of MutYH and MTH1 remained constant, the amounts of Ogg1 decreased with increasing differentiation, concomitant with an accumulation of 8-oxo-G detected in those cells. Taken together, in this study at least, ES cells seemed more resistant to oxidative stress than differentiated cells.

Commonly, the mutagenic potential of oxidative DNA damage is thought to be most important during DNA replication and cell division. However, it was shown in *Saccharomyces cerevisiae* (*S.cerevisiae)* that mutations arising during times of cell cycle-arrest stemming from endogenous oxidative stress might considerably contribute to aging and carcinogenesis (93). A distinct increase in ROS in the course of starvation-induced cell cycle-arrest and an accumulation of apurinic/apyrimidinic sites (AP sites) and DSBs under these conditions could be observed, supporting the hypothesis that the incidence of spontaneous frameshift mutations in a cell cycle-arrested state is considerably influenced by oxidative stress.

Recently, instead of only concentrating on the repair of single, isolated lesions occurring in DNA, the attention of researchers has also been shifting toward investigation of the repair of so-called 'clustered DNA lesions'. A clustered DNA lesion is defined as the occurrence of two or more damages in the DNA within one to two helical turns, usually only induced by ionizing radiation and certain chemicals in this non-random way. Such lesion clusters are more toxic to cells than isolated lesions and also are the reason why ionizing radiation and bleomycin sulfate are more efficient in killing cells than damage produced by ROS as $H_2O_2$. The question what happens if instead of single lesions 8-oxo-G is present in a whole cluster of DNA damage was addressed in an interesting paper by Eccles *et al*. For this study, synthetic oligonucleotides with three-lesion clusters containing AP sites and 8-oxo-G lesions were utilized. They found that AP sites close to an 8-oxo-G lead to the formation of DSBs, probably arising via lesion processing rather than stalled replication in cycling cells (94). This study provides evidence that it is not only the direct formation of DSBs that has implications for cell survival but that non-DSB clusters can be converted into DSBs during processing and attempted repair. For more details concerning the field of clustered DNA lesions in yeast and mammalian cells see the recent review by Sage and Harrison (95).

New insights regarding the occurrence and repair of oxidation damage in telomeric DNA, the extremities of chromosomes, have been gained recently. Lu *et al* showed that Ogg1 also repairs 8-oxo-G lesions occurring in the G-rich telomeric DNA by BER, and ablation of Ogg1 in *S.cerevisiae* leads to an increase in 8-oxo-G in telomeric DNA and induces telomere lengthening by telomerase/Rad52 mediated homologous recombination (96). These results suggested that 8-oxo-G may perturb telomere length equilibrium by disturbing telomere length maintenance and propose that interfering with telomere length homoeostasis may be one of the mechanisms by which oxidative stress damages the genome. Wang and colleagues studied the influence of 8-oxo-G repair by Ogg1 on mammalian telomeres in *ogg1*$^{-/-}$ mouse tissues and primary MEFs under different oxidative culturing conditions (97). When cultivated at 3% oxygen (hypoxic conditions), telomere lengthening was observed, whereas telomeres seemed to shorten in hematopoietic cells and primary MEFs cultivated under normoxic conditions (20% oxygen) or in the presence of an antioxidant. Additionally, telomere length abnormalities, telomere sister chromatid exchanges, increased telomere SSBs and DSBs, and preferential telomere losses of the lagging- or G-strand were observed, indicating that 8-oxo-G damage can arise in telomeres, affecting

length homeostasis, recombination, DNA replication, and DNA break repair. Along the same lines, a study examining the susceptibility of telomeric DNA to oxidative base damage demonstrated that telomeric TTAGGG repeats were more prone to oxidative base damage and repaired less efficiently than non-telomeric TG repeats *in vivo* (98). They showed that the activity of Ogg1 was similar in telomeric and non-telomeric double-stranded substrates and that it was not impaired by telomere repeat binding factors Trf1 and Trf2. However, in some telomeric structures (e.g., fork-opening, 3'-overhang, and D-loop) 8-oxo-G was less effectively excised by Ogg1, depending on its position within these substrates. Collectively, these data indicated that the sequence context of telomeric repeats and certain telomeric configurations may contribute to telomere vulnerability during oxidative DNA damage processing.

The question if and how transcription is affected by the presence oxidative DNA damage within a DNA segment that is transcribed was addressed by Khobta and co-workers. They described that 8-oxo-G does not constitute a barrier to transcription itself, concomitant with the notion that 8-oxo-G is not regarded as a blocking lesion *per se (99)*. However, 8-oxo-G was converted into a transcription-blocking damage by the presence of Ogg1 even if present only in the non-transcribed strand, suggesting that the interruption of transcription was induced by the formation of BER intermediates. As also the non-blocking lesion uracil was found to induce a similar blockage independently of Ogg1 activity. The blockage of transcription could therefore be a common consequence of various DNA base modifications. As Cockayne syndrome (CS) cells display impaired Transcription-coupled repair (TCR), Cramers *et al* examined the question whether TCR plays a role in IR-induced oxidative DNA damage repair or if CS plays a role in transcription elongation after irradiation (100). While they could not find any evidence for a key role of TCR in repair of IR-induced oxidative damage, a reduction of overall repair of oxidative damage could be seen in Cockayne syndrome A (CSA) and Cockayne syndrome B (CSB) cells, as assessed by repair replication. This suggests that impaired repair of oxidative lesions throughout the genome may contribute to the CS phenotype. Also, CSB could be shown to play a significant role in mitochondrial BER regulation, as analysis of 8-oxo-G, uracil, and 5-OH-uracil BER incision activities were reduced in CSB-deficient cells compared to wild-type cells (101). These results in total suggested that CSB plays a role in mitochondrial BER by helping to recruit, stabilize, and/or retain BER proteins in repair complexes associated with the inner mitochondrial membrane. Clauson *et al* reported in an interesting publication that both TCR and Nucleotide-excision repair (NER) are utilized by *E.coli* to repair 8-oxo-G and uracil lesions (102). The relative level of recognition of these two lesions by BER and NER suggested that components of either pathway could be used by TCR for lesion removal, depending on their availability. Concluding, these findings demonstrated a dynamic flexibility of DNA repair pathways in the removal of non-bulky DNA lesions in prokaryotes. It remains to be elucidated, how these repair pathways cooperate in mammals in the repair of 8-oxo-G. Another study analyzing *E.coli* showed that the 8-oxo-G repair deficiency that is compromised in *E.coli* strains deficient for mutT, mutY and mutM (the homologs for MTH1, MutYH and Ogg1) can be rescued by NorM, a member of the multidrug and toxin extrusion family of efflux pumps (103). NorM was found to reduce the level of intracellular ROS, thus protecting the cell from specific ROS when the 8-oxo-G repair system cannot cope with the damage.

An attempted answer to the question, how much eventual 8-oxo-dGTP pools contribute to 8-oxo-G induced mutagenesis, was given by Wilson and co-workers (104). The analysis of recent structures of Pols from several families with 8-oxo-G in the nascent base pair binding pocket revealed that flexibility around the template-binding pocket can permit 8-oxo-G to assume either an anti- or a syn-conformation, and thus code for incorporation of C or A, respectively. In contrast, the binding pocket for the incoming nucleotide does not have this flexibility so that insertion of 8-oxo-dGTP opposite C is strongly discouraged. Therefore, it can be assumed that 8-oxo-dGTP incorporation into DNA by Pols is an event that probably rather rarely occurs in a cell.

Saha *et al* were able to show that Ogg1 expression is inducible when cells experience oxidative stress. More precisely, BRCA1, a tumour suppressor associated with breast cancer development, induces repair of oxidized DNA by stimulating Ogg1, the DNA glycosylase NTH1, and Ape1 in human breast carcinoma cells by increasing the expression of those enzymes (105). This stimulation was found to be dependent on the transcription factor Oct1, and the expression of all those enzymes was inducible by oxidative stress caused by $H_2O_2$ treatment of cells.

Regarding the question, which Pol is the main responsible for dealing with 8-oxo-G DNA damage, a crystallographic study assessing a complex of Pol ι with 8-oxo-G was performed. The results showed that the exceptionally narrow active site of Pol ι can prevent the dual coding properties of 8-oxo-G by inhibiting the syn/anti conformational equilibrium (106). The authors claim that this work provides the structural mechanism of high-fidelity 8-oxo-G replication by a human Pol. However, the authors don't comment in any way on the data showing that Pol λ is at least 1200-fold more faithful in bypassing 8-oxo-G *in vitro* (7) and acts on sites of oxidative DNA damage *in vivo (20)* or on all the other

experimental data indicating Pol η might also play a role in 8-oxo-G repair (e.g. (107, 108)). It remains to be seen how much of 8-oxo-G repair is really attributable to the action of Pol ι.

Monoubiquitination of PCNA at K164 by the coordinated action of the E2 Rad6 and the E3 ubiquitin ligase Rad18 takes place following treatment of cells with DNA damaging agents that induce bulky, DNA replication fork blocking lesions (109). In a very recent study by Kannouche *et al* it was found that PCNA becomes rapidly and transiently monoubiquitinated in response to oxidative stress and that this takes place independently of the S phase (110). Monoubiquitination of PCNA is further promoted by the heterodimer Msh2/Msh6 in a pathway independent from the 'canonical' MMR - and subsequently recruits Pol η to the chromatin. This suggests the existence of a mechanism in which Pol η coordinates with Msh2/Msh6 to remove complex oxidative DNA lesions from chromatin in human cells. This study also underlines the intricate network and the multiple layers of redundancy in place regarding the repair of oxidative DNA damage *in vivo* that slowly begin to emerge.

## DNA Polymerase λ

Recent data from the Wilson lab using *Pol*λ-/- *Pol*β-/- double knockout MEFs suggested that both of these Pols mediate overlapping as well as independent roles in BER in MEFs (111). The double knockout cells showed a hypersensitivity to alkylating and oxidizing agents. The contribution of Pol λ to single-nucleotide BER was only modest, but both Pol λ and Pol β were shown to interact with the upstream DNA glycosylases involved in the repair of alkylated as well as oxidized DNA bases, indicating these interactions to be important in coordinating the Pol choice during BER. Pol λ is the only member of the X family in plants. A study by Amoroso et al, addressed Pol λ's role in the plant *Arabidopsis thaliana (A. thaliana)*, where they could show that Pol λ is as efficient in performing error-free TLS past 8-oxo-G as its mammalian homolog (112). Plants overexpressing or silenced for Pol λ showed altered growth phenotypes. Furthermore, they could show that Pol λ interacts only with PCNA2, one of the two PCNA genes present in *A. thaliana*, and that this interaction enhances the fidelity and efficiency of Pol λ in TLS. Interestingly, further underlining the importance of Pol λ for *A. thaliana*, the promoter of the POL L gene could be activated by UV. A collaboration with the Villani group (see the original manuscripts attached below), puts forward experimental evidence for the involvement of Pol λ, together with Pols β and η in the bypass of AP sites in vitro (38). Interestingly, the nature of the template downstream to the lesion seemed to dictate the choice of the Pol used for bypassing an AP site. Whereas the presence of a downstream primer did not influence the bypass efficiency by Pol η, it was required for the bypass of the AP site by Pols λ or β. Moreover, the N-terminal BRCT and P/S-rich domain of Pol λ seemed to be prerequisite for the ability of Pol λ to perform TLS. In conclusion, this study indicated the existence of a Pol switch at an AP site from the replicative Pol ε to the repair Pols λ and β.

## MutYH DNA glycosylase

A recent study by Molatore *et al* investigating several mutations of MutYH that are found in MAP patients (137insIW, R171W, E466del, Y165C and G382D) by using a new cell-based analysis with *mutyh*$^{-/-}$ MEFs demonstrated that all of the tested MutYH variants were dysfunctional in BER (113). This was corroborated by *in vitro* data, with the only exception of the G382D mutant, which showed a glycosylase activity very similar to the wild-type protein. Surface plasmon resonance studies by D'Agostino *et al* to assess the binding affinity towards an A:8-oxo-G substrate revealed a severe reduction for MutYH variants R171W, E466del and Y165C, which was associated with an impairment of the glycosylase activity, but only a slight decrease in binding by the137insIW and G382D mutants (114). Suzuki *et al* investigated the repair of 8-oxo-G in DNA and 8-oxo-dGTP in 293T cells by OGG1, MutYH, NTH1, and NEIL1 using supF shuttle plasmids (115). The knockdown of all of them resulted in a significant increase in CG → AT transversions caused by the C:8-oxo-G pair in the shuttle plasmid. Furthermore, the knockdown of MutYH, but not the other glycosylases, resulted in a reduction in AT → CG transversions induced by 8-oxo-dGTP. These results indicate that all of the tested DNA glycosylases suppress mutations caused by C:8-oxo-G in DNA and that MutYH in particular suppresses mutations induced by C:8-oxo-G in DNA, but enhances those generated by 8-oxo-dGTP. Another study addressing Synthetic sickness/lethality (SSL) by the combined deficiency of Msh2 with Pol β or MLH1 with Pol γ found that the SSL phenotype could be rescued by the knockdown of MutYH, suggesting that lethality could be caused by the formation of lethal DNA breaks upon 8-oxo-G accumulation (116).

The link between increased cancer risk associated with inflammatory bowel diseases compared the inflammatory response of wild-type and *mutyh*$^{-/-}$ mice to oxidative stress by dextran sulfate sodium was assessed by Casorelli *et al* (117). By inducing ulcerative colitis, they found that *mutyh*$^{-/-}$ mice showed less severe lesions, lymphoid hyperplasia

and a significant reduction in Foxp3(+) regulatory T cells. Their findings indicate that MutYH could play a major role in maintaining intestinal integrity by affecting the inflammatory response.

Mass spectrometric data analysing posttranslational modifications (PTMs) of MutYH showed that an *in vivo* phosphorylation site is present at S524, which is located in the C-terminal 8-oxo-G recognition domain within the PCNA binding region (118). Characterization of the phosphomimetic S524D and phosphodeficient S524A mutants suggested that this residue may play an important role in MutYH regulation *in vivo* by altering stability and A:8-oxo-G mismatch affinity.

## Posttranslational modifications

The term PTM refers to the chemical modification of a protein by the addition of chemical groups to its polypeptide chain. Such moieties can be phosphate, acetate, sulfate or alkyl groups, lipids, carbohydrates, polypeptides and others that are covalently attached, to name only a very few of them. Many proteins undergo co- and/or post-translational modifications during their lifespan. Knowledge of these modifications is of high importance, because they can profoundly alter physical and chemical properties of the protein they are attached to.

As already mentioned in the review above, PTMs are believed and have been shown to greatly influence the stability, localization, regulation and activity status of many proteins involved in the repair of oxidative DNA damage. In order to understand more about how they regulate and impact on proteins involved in DNA repair, the focus of this work was put on two PTMs, namely phosphorylation and and ubiquitination.

### Phosphorylation as posttranslational modification

Reversible protein phosphorylation is a key event in cellular regulation and it was for the discovery of this mechanism that the Nobel Prize in physiology or Medicine 1992 was awarded jointly to Edmond H. Fischer and Edwin G. Krebs *"for their discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism"*.

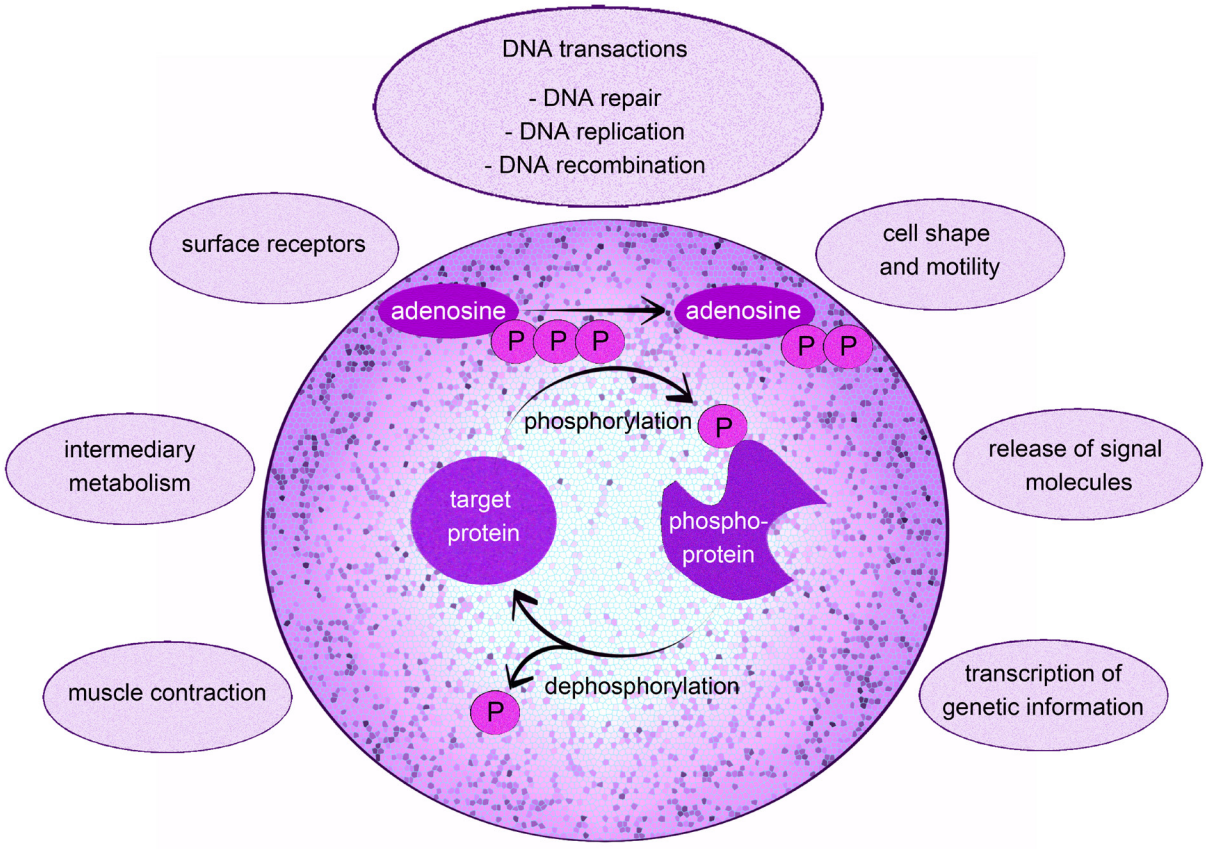


***Figure 2: Scheme of the protein phosphorylation and -dephosphorylation reaction.***
Phosphorylation is brought about by the transfer of a Phosphate moiety (P) from ATP to a target protein by protein kinases, thereby changing the functions of the taret protein. Dephosphorylation is the reverse mechanism and is catalysed by protein phosphatases. Regulation of proteins by phosphorylation is involved in a multitude of processes, as cell surface receptor activity control, cellular metabolism, contraction and relaxation of muscles, release of hormones and nerve signal molecules, transcription of genetic information, protein synthesis by the ribosomes and cell shape and motility. Also, DNA transactions as DNA repair, DNA replication and DNA recombination have been shown to be regulated by phosphorylation (Adapted from the Nobel lecture by Fischer and Krebs).

Phosphorylation is the addition of a phosphate ($PO_4^{3-}$) group to a polypeptide or protein by enzymes called protein kinases. Those kinases move a phosphate group from ATP to the target protein (*Figure 2*). The reverse mechanism – the

removal of a phosphate group from a target protein to revert it back to its original state – is termed dephosphorylation and is catalysed by protein phosphatases. Regulation of proteins by phosphorylation plays an important role in both prokaryotic and eukaryotic organisms (119-121). An enormous array of cellular functions are tightly regulated by and heavily rely on phosphorylation. Many enzymes and receptors are switched forth and back between their "on" and "off" state by phosphorylation and dephosphorylation, because the addition and removal of such a moiety frequently results in a conformational change in the structure of the modified target protein. This change is often brought about by the introduction of the negative charge of the $PO_4$ moiety to a polar or uncharged group, which can turn a hydrophobic part of the protein into an extremely hydrophilic one. Phosphorylation commonly occurs on Serine (S), Threonine (T) or Tyrosine (Y) residues in eukaryotic proteins. In prokaryotes, it can take place on Histidine (H), Arginine (R), or Lysine (K), all of which positively charged amino acids.

The progression of the cell cycle is tightly regulated by a family of Cdks. Members of this family of proteins phosphorylate and thereby activate other proteins. The activity of these target proteins is pivotal for further events taking place to drive a cell in a coordinated manner through the entire cell cycle (122). In order to become active, Cdks require to associate with their respective interaction partners, the cyclins, and to be phosphorylated by a Cdk activating kinase at a conserved T residue (122). Every single phase of the cell cycle is characterized by the expression of a different subset of Cdk/cyclin complexes that phosphorylate and regulate downstream substrates. In vertebrates these are Cdk4/6/cyclinD throughout the G1 phase, Cdk2/cyclinE at the G1/S boundary, Cdk2/cyclinA during S phase and Cdk1/cyclinA as well as Cdk1/cyclinB during the G2/M transition. The diversity of targets that those complexes phosphorylate is far from being completely identified and the entire functional outcome of these events is understood so far in only a minority of cases. Future phosphoproteome analysis will give further insight into this very complex regulatory cellular network.

## Ubiquitination: the cell's way to dispose of waste – and more

Posttranslational modification of proteins by conjugation of ubiquitin (ubiquitination) is important for many aspects of eukaryotic life. As for the aforementioned phosphorylation, ubiquitin is reversibly attached to the target protein, making it ideal for regulatory purposes. Ubiquitin is a 76 amino acid polypeptide that can be attached to a K residue in the target protein, and can also, in contrast to phosphorylation, be attached to another ubiquitin residue, thus giving rise to the formation of mono-, di-, and polyubiquitin chains that can even result in branched tree-like structures.

The conjugation of ubiquitin is performed in an ATP dependent mechanism and involves the concerted action of ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3), all of which have been thoroughly reviewed (123-125). Those three classes of enzymes have to work together in a very organised way to perform their task of attaching ubiquitin to the target. Some of them also possess elongating activities that support the generation of polyubiquitin chains. Polyubiquitination occurs through addition of a new ubiquitin to on any of the seven K residues (K6, K11, K27, K29, K33, K48 or K63) or the N-terminal first methionine of the ubiquitin monomer. Thus, ubiquitin can be connected to long chains by at least eight different linear linkages, as well as by a range of atypical chains formed by heterologous, forked or mixed conjugation.

The substrate specificity of the reaction is either provided by the E3s, that typically have substrate-binding sites, or by a combination of E3s with E2s. The only two E1 present in human cells are standing in gross contrast to the abundance of the E2s and E3s: to achieve a high regulatory potential and specificity of the ubiquitination reaction, mammalian cells express a multitude of E2s (estimations are around 30 in humans) and even more E3s (estimated more than 300 in humans). Specialized ubiquitin-binding domains in interacting proteins recognise the ubiquinated proteins and allow non-covalent protein-protein interactions with the ubiquitin itself or the region around the ubiquitin attachment to take place. The reverse reaction of ubiquitination is termed deubiquitination and is accomplished via the action of deubiquitinating enzymes, a family of isopeptidases that removes the ubiquitin moieties from the target proteins.

Ubiquitination has been implicated to be important for the regulation of different aspects of cellular physiology. Protein degradation, DNA repair, receptor endocytosis, apoptosis and autophagy are just a few of them. Nevertheless, the first, and in the context of this work most important feature to be attributed to ubiquitination, is its function as a signal for proteasomal degradation. The process of ubiquitin-mediated proteasomal degradation has been reviewed in (126-129). Following the marking of a protein with ubiquitin, the target is selectively degraded in an ATP-dependent process by an enormous complex called proteasome, whereupon free ubiquitin and small peptides are released. Proteasomes in eukaryotic cells are huge multiprotein particles consisting of 50 subunits totaling 2.4 mDa and form up to 1% of the cellular protein residing in the cytosol and the nucleus. As a consequence, only one proteasome is present in the nucleus

and cytosol per cell.

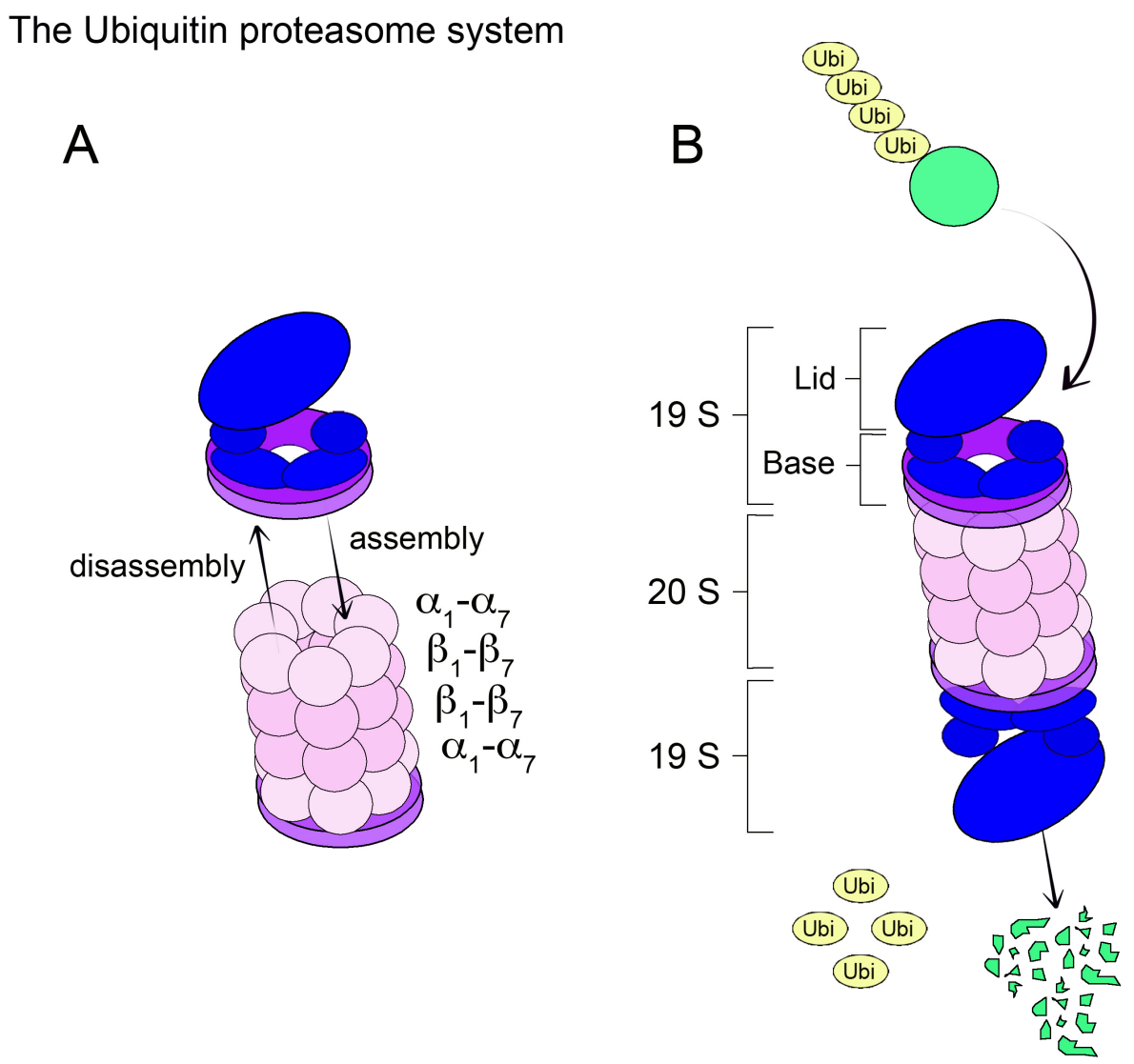


***Figure 3: The 26S proteasome.***
The proteasome is built of two subcomplexes, the core 20S catalytic particle and the 19S regulatory particle, the protein components of which are variable. A) The 20S particle consists of 4 heptameric rings, two α-rings and two β-rings that form a channel. The central two rings lining this channel are made of the β-subunits β1 - β7, while the two rings at either side of those comprise of α1 - α7 subunits. This channel is topped on one end (often also on both ends) by the 'lid'-like structure of the 19S regulatory particle. The substrate binding occurs probably via the polyubiquitin chains on the target protein to the 19S subunit. The 19S subunit then inserts the substrate via an open 'door' into the α-ring of the 20S. At the center of the 20S particle lies the proteolytic chamber of the complex. Depending on the composition of the lid, different target proteins will be recruited to the proteolytic channel. As mentioned, the 19S particle is of variable composition and associates with the 20S particle in an assembly/disassembly reaction. The assembly and disassembly of the two subunits can be controlled by a variety of factors. B) A target protein marked by ubiquitination is inserted into the channel, where it is broken down into small peptides by three pairs of proteolytically active β-subunits residing in the two β-rings. In addition to mediating the substrate recognition, the 19S particle also contains deubiquitinating enzymes that allow the recycling of ubiquitin. (Adapted from Weissmann *et al* (129)).

The 26S proteasome is composed of a so-called core 20S core particle, which allows the digestion of proteins to short peptides, and one to two 19S regulatory particles (*Figure 3*). The 19S regulatory particles are responsible for substrate recognition and transport into the core particle. The 20S particle resembles a tube that is formed by four stacked rings surrounding a central cavity. Inside this central chamber lie the proteolytic sites of the β subunits, which face the central den, allowing protein digestion to be clearly isolated from the surrounding cytosol. Protein digestion is brought about

by the action of two sites cleaving after hydrophobic residues, two others cutting after acidic residues and two more that chop the protein after basic residues. Thus, proteasomes are capable of cutting most types of peptide bonds. The assembly and disassembly of the proteasome itself and the degradation of some of its subunits are processes that are also regulated. Even though the proteasome is generally regarded as a very stable protein complex, the entire proteasome or its subcomplexes are probably subject for degradation by the lysosome via the microautophagy pathway. Different factors as ATP, proteasome inhibitors and ubiquitinated substrates control the assembly of the proteasome and the disassembly of the proteasome to its regulatory particle and core particle subunits can be induced by different stress stimuli, such as oxidative stress and cellular starvation. The regulatory particle can be further disassembled into its individual subunits, which are probably also degraded by ubiquitin-dependent proteasomal degradation.

Maintenance of balanced levels of proteins is of pivotal importance for the correct functioning and survival of each cell. It is clear that it could prove detrimental for many functions if either too much or too little of certain protein components were present. For instance, failing to degrade Cdks in time would lead to a stall, or worst case, even breakdown of the cell cycle. Also, one can imagine that uncontrolled levels of a DNA repair protein could lead to the accumulation of deleterious mistakes in the genomic DNA. It is this regulation that is mediated by the ubiquitin-dependent proteasomal degradation. Importantly, the degradation of a target protein can be regulated and influenced in turn by other PTMs, such as phosphorylation. This crosstalk of different PTMs adds a whole different layer of complexity to the question how, when and to what end proteins are regulated in the cell. In conclusion, regulation of cellular protein levels by the ubiquitin-proteasome system is an important feature of healthy cells and that the interplay between ubiquitination of a protein and other PTMs constitutes a complex field of investigation that still has a lot left to explore in order to understand its full implications.

## Ubiquitination and DNA repair

The role of ubiquitination in DNA repair has been investigated extensively already in the context of DSB repair (reviewed in (130)). In short, upon phosphorylation by ATM, MDC1 gets activated and triggers the recruitment of the E3 ligase RNF8. Together with Ubc13, RNF8 ubiquitinates the histone protein H2A, which in turn is a signal that recruits an additional E3 ligase RNF 168. Further ubiquitination at the DSB site by RNF168 leads to an amplification of K63-linked ubi-H2A around the DSB, coordinating recruitment and timing of further repair proteins that subsequently repair the break.

TLS of lesions, such as UV lesions and cisplatin-induced intrastrand GG crosslinks, that cannot be bypassed by the replicative Pols has been found to be dependent on ubiquitination, as has been reviewed in (131). More precisely, upon encounter with a blocking DNA lesion, PCNA gets ubiquitinated by an E2/E3 complex consisting of Rad6 and Rad18. This monoubiquitination takes place at K163 of PCNA and serves as recruiting signal for damage-tolerant translesion Pols, such as Pol η. These Pols then perform TLS and are subsequently replaced by the replicative Pols again, which resume the polymerization of long stretches of DNA. In addition to being monoubiquitinated, PCNA has also been shown to undergo polyubiquitination by the E2 Ubc13-Mms2 and the E3 Rad5 on the same residue in yeast. Most probably, this polyubiquitination leads to the activation of an error-free repair pathway relying on template-switching, the exact mechanisms of which remain unclear.

Regarding the regulation of components of the BER machinery by ubiquitination, only little is known so far. Dianov and his group showed that components of the BER machinery are targeted for destruction by the E3 ubiquitin ligase CHIP under physiological, unstressed cellular conditions (132). CHIP is a protein that is involved in controlling the cellular levels of various proteins, such as p53. When DNA damage occurs, these components are stabilized to increase the cellular capacity to perform BER. More precisely, proteins such as Pol β, XRCC1 and DNA ligase III are stabilized when bound to chromatin, thus forming an active repair complex. However, when they are not attached to chromatin and thus are not engaged in DNA repair, those components are polyubiquitinated by the concerted action of the E3 ubiquitin ligase CHIP and consequently degraded. Sobol suggested in a ‘‘preview’’ in the same issue of Mol. Cell, that ‘‘the next goal is now to assess the crosstalk between PTM’s, the ability to form productive repair complexes and the stability of these complexes’’(133). He put forward the concept that a single PTM, such as phosphorylation, might influence the targeted enzyme’s function positively or negatively and also constitute a signal for further PTM’s.

Follow-up work on the regulation of BER proteins in the cell by the Dianov lab showed that the E3 ligase Mule also ubiquitinates Pol β (134). They unveiled the existence of a fine-tuning mechanism in place to govern the cellular levels of Pol β in order to coordinate the steady-state levels of BER enzymes with the genomic DNA damage background of

an individual cell. This mechanism involves the action of the E3 ligase Mule which monoubiquitinates Pol β, whereby it is subsequently targeted for polyubiquitination by CHIP. This regulation of cellular Pol β levels by Mule is thought to be responsible for effective responses to minor fluctuations in endogenous DNA lesions.

In summary, although data on the regulation of BER proteins by ubiquitination exist, the mechanisms are still far from being completely understood and much more work is needed to appreciate its full implications. Also, the decoding of the cross-talk of different PTMs will be of high interest in the near future in order to unveil the mechanisms by which they coordinate the abundance, the activity and the localization of the different repair complexes within the cell.

# Aim of the thesis:

### Understanding the regulation of cellular DNA Polymerase λ levels by phosphorylation and ubiquitination

The main focus of the work presented in this thesis was to elucidate the mechanisms that regulate the protein levels and activity of Pol λ in the cell. Clinical evidence that the regulation of Pol λ as a rather promiscuous DNA repair enzyme is important comes from a study performed by Ohba *et al*. They showed that the expression of Pol λ in human bronchiolar epithelia correlates with the amount of habitual smoking (135). This is in line with the findings by Albertella *et al* who found Pol λ, among other Pols, to be more than two-fold overexpressed in 24% of cancer samples compared to their normal tissue counterparts (136).

## Background

The first data regarding the regulation of Pol λ *in vivo* came from a study by Frouin *et al* showing that Pol λ interacts with and is phosphorylated by Cdk2 *in vitro* in the P/S-rich domain (137). While this phosphorylation did not affect Pol λ's polymerase activity, the phosphorylation was decreased by an interaction of Pol λ with PCNA. Finally, it was shown that Pol λ is phosphorylated *in vivo* during the cell cycle and that the phosphorylation pattern mimicked the pattern of Cdk2/CyclinA's fluctuation during the cell cycle. Follow-up work by Wimmer *et al* then led to the identification of several phosphorylation sites of Pol λ. Experiments with phosphorylation-defective mutants suggested a critical function for the residue T553 in the maintenance of Pol λ's stability throughout the cell cycle and specifically during late S and G2 phase (138). A loss of phosphorylation of the T553 residue lead to increased ubiquitination and proteasomal degradation of Pol λ. Thus, the stabilization of Pol λ during the S and G2 phases is likely to enable it to conduct DNA damage repair at this stage of the cell cycle. Since it is known that all the replicative Pols preferentially incorporate an A opposite 8-oxo-G instead of the correct C, these findings are in line with a putative role of Pol λ in post-replicative repair of 8-oxo-G.

So far only little is known how the BER components and their activity are regulated in the cell. PTMs, such as phosphorylation, acetylation, sumoylation, methylation and ubiquitination provide an intriguing possibility of regulation of those proteins (reviewed in (139)). Recently, the group of G. Dianov found that Pol β, XRCC1 and DNA ligase III, all members of BER, are stabilized when participating in a chromatin-bound active repair complex (132). On the contrary, when they are in a 'soluble', not chromatin-bound form and thus not participating in DNA repair, they become polyubiquitinated by the E3 ligase CHIP. This polyubiquitination targets the non engaged BER proteins for proteasomal degradation.

## Aim

The idea that PTMs like phosphorylation might fine-tune the affinity of Pol λ to an E3 ubiquitin ligase, thus determining whether the protein is targeted for proteasomal degradation or recruited to chromatin to fulfil its function in DNA repair, was very intriguing. The aim of this thesis was therefore to take a closer look at the regulation of Pol λ *in vivo* by ubiquitination and phosphorylation. It led to the formulation of two core questions that constitute the basis of the presented thesis: firstly, we wanted to identify the E3 responsible for the degradation of Pol λ *in vivo* and, secondly, to see if and how this degradation could be regulated by phosphorylation of Pol λ by Cdk2/CyclinA. The results of this study can be found in the manuscript 'Regulation of oxidative DNA damage repair by DNA Polymerase λ and MutYH by cross-talk of phosphorylation and ubiquitination' that is attached below (p. 46).

# Original manuscripts

## "Ubiquitylation of DNA Polymerase λ"

**Enni Markkanen***, Barbara van Loon*, Elena Ferrari and Ulrich Hübscher
* joint first authors

In the following manuscript in which I was a joint first author, early data concerning the ubiquitination of Pol λ by CHIP, one of the two E3 identified to ubiquitinate Pol λ *in vitro*, are presented. Furthermore, a possible link between induction of Pol λ levels and the E3 Mule upon treatment of cells with $H_2O_2$ as agent that causes oxidative stress was shown.

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Review

# Ubiquitylation of DNA polymerase λ

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ABSTRACT

**DNA polymerase (pol) λ, one of the 15 cellular pols, belongs to the X family. It is a small 575 amino-acid protein containing a polymerase, a dRP-lyase, a proline/serine rich and a BRCT domain. Pol λ shows various enzymatic activities including DNA polymerization, terminal transferase and dRP-lyase. It has been implicated to play a role in several DNA repair pathways, particularly base excision repair (BER), non-homologous end-joining (NHEJ) and translesion DNA synthesis (TLS). Similarly to other DNA repair enzymes, pol λ undergoes posttranslational modifications during the cell cycle that regulate its stability and possibly its subcellular localization. Here we describe our knowledge about ubiquitylation of pol λ and the impact of this modification on its regulation.**


## 1. DNA polymerase λ, a multitask repair enzyme

Seven DNA polymerase (pol) families have been defined based on sequence homologies. They are called family A, B, C, D, X, Y and reverse transcriptase (RT) [1,2]. The eukaryotic pols can be divided into the five families A, B, X, Y and RT. Pol λ belongs to the X family of DNA pols, which comprises pol β, pol μ and terminal deoxyribonucleotidyl transferase in addition to pol λ [1].

Pol λ is the product of the POLL gene, localized on chromosome 10 in humans and chromosome 19 in mice and is composed of 575 amino acid residues (the murine form having 573 residues) [3]. The C-terminal part of pol λ shows the typical 'right-hand' folding with a palm, finger, thumb and an additional 8 kDa dRP-lyase containing subdomain. The first 230 N-terminal amino acids compose the BRCT and the proline/serine rich domain [4,5] (Fig. 1A). The BRCT domain is believed to be important for protein/protein interactions with components of non-homologous end-joining (NHEJ) such as XRCC4/DNA ligase IV [6]. A possible role of the non-enzymatic proline/serine-rich domain might be modulation of pol λ fidelity, since pre-steady state kinetic studies suggested that this domain contributes to its accuracy [7]. Pol λ possesses multiple activities (reviewed in [2]): in addition to template dependent DNA polymerization it displays dRP-lyase, as well as template-independent terminal deoxynucleotidyl transferase and polynucleotide synthetase activities (Fig. 1B). Also, it efficiently adds DNA bases to a RNA primer [8]. The dRP-lyase activity of pol λ hints towards an involvement in base excision repair (BER) [9] and in vitro experiments suggest that pol λ participates in double strand break DNA repair (DSBR) via NHEJ [10]. In addition, biochemical evidence supports a prominent role of pol λ in the correct repair of oxidative DNA lesions such as 8-oxo-guanine [11–13] and 2-hydroxy-adenine [14], and it seems to perform those functions in cooperation with the auxiliary proteins replication protein A (RP-A) and proliferating cell nuclear antigen (PCNA) (see below). Only in the presence of these two auxiliary proteins the remarkably accurate incorporation opposite 8-oxo-G can be achieved, since the bias of C versus A incorporation increases over 1200 (see Ref. [12] and Table 1 therein). Moreover, pol λ isolated from calf thymus tissue was shown to efficiently bypass apurinic/apyrimidinic (AP) sites [15]. A polymorphic variant of pol λ (R438W) was found to affect the homologous recombination (HR) pathway and sister chromatin exchanges, suggesting that pol λ also has a function in HR [16].

Biochemical studies in Suo's laboratory suggested that an increase in gap size results in lower accuracy for pol λ [17]. The observed decrease in the fidelity appears to be regulated by non-enzymatic N-terminal domains (also see above). Moreover, dCTP was the preferred misincorporated base for full-length pol λ and its N-terminal domain truncation mutants. Their results also indicated that pol λ catalyzes nucleotide incorporation with the

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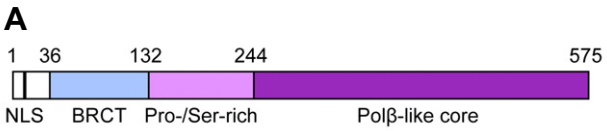


B

| Activities | Functions |
|---|---|
| **DNA polymerase** | DNA synthesis (TLS, BER, NHEJ, HR) |
| **Terminal transferase** | V(D)J recombination, NHEJ |
| **dRP lyase** | Short-patch BER |

**Fig. 1.** Structure and functions of DNA polymerase λ. For details see text and references therein.

**Table 1**
Post-translational modifications of BER proteins.[a]

| Protein | Modification |
|---|---|
| TDG | Sumoylation, acetylation |
| OGG1 | Phosphorylation, acetylation |
| APE1 | Phosphorylation, acetylation, ubiquitylation |
| MUTYH | Phosphorylation |
| Pol β | Phosphorylation, acetylation, methylation, ubiquitylation |
| Pol λ | Phosphorylation, ubiquitylation |
| Pol δ | Phosphorylation, sumoylation, ubiquitylation |
| Pol ε | Phosphorylation |
| PCNA | Phosphorylation, acetylation, sumoylation, ubiquitylation |
| Fenl | Phosphorylation, acetylation |
| DNA lig I | Phosphorylation |
| DNA lig III | Phosphorylation |
| XRCC1 | Phosphorylation, sumoylation, ubiquitylation |

[a] For details see [31,32] and references therein.

highest combination of efficiency and accuracy when the DNA substrate contains a single-nucleotide gap.

Non-overlapping functions of pols μ, λ, and terminal deoxyribonucleotidyl transferase have been described during immunoglobulin V(D)J recombination in vivo [18]. Pol λ knockout mice are viable and fertile and display a normal hypermutation pattern [19]. Pol $\lambda^{-/-}$ mouse embryonic fibroblasts were shown to be more sensitive to oxidative DNA damage and this phenotype was further enhanced when combined with inactivation of the closely related enzyme pol β, suggesting backup functions these two proteins in the repair of DNA oxidative lesions [20,21]. Furthermore, ionizing radiation sensitivity has been seen in pol λ knockout cells [22].

The pol X family is well characterized on the structural level (reviewed in [23]). Figuratively speaking a pol resembles a human right hand consisting of a palm fingers and a thumb (reviewed in [24]). In general, structural motions of the whole enzyme are observed upon binding of the dNTP's into the active site located in the palm domain. In contrast to this general feature of pols, pol λ was found not to require that subdomain motion (e.g. in fingers and thumb) for catalysis [25], thus making this enzyme particular within all the pol families. When the binary (pol λ/template/primer) and the ternary (pol λ/template/primer/dNTP) complexes were compared, it was found that the essential Asp427, Asp429 and Asp490 possess the same positions whether or not an incoming dNTP was present. Key amino-acids are the Tyr505 and the Phe506 [26] that form the contact with the minor groove of the correctly positioned DNA [25]. Further, it was shown that pol λ can generate single-base deletions during DNA synthesis [27]. This feature was explained on the structural level to be due to DNA strand repositioning induced by the dNTP catalysis, thus controlling the strand slippage [28]. Finally, when the catalytically active form of pol λ was bound to the template/primer with an extrahelical template nucleotide upstream of the active site, pol λ generated strand slippage mutations [29]. In other studies using a mass spectrometry-based protein footprinting approach a solution-phase protein conformational change in pol λ was found [30]. The discrepancy between this observation and the previous structural studies might be due to the fact that the crystallographic structural studies were performed with the catalytic domain only, while the solution studies were carried out with full-length pol λ also containing the BRCT and the proline/serine-rich domains.

In summary, pol λ is a multifunctional enzyme with important functions in BER, NHEJ and translesion DNA synthsis (TLS), the pathways that evolved to reduce the mutational burden in a cell. Unique structural features of pol λ reflected in its enzymatic activity, might be essential for its role as a multifunctional DNA repair pol.

## 2. Regulation by posttranslational modifications of BER proteins in general and DNA polymerase λ in particular

At present little is known about regulation of BER and its components in the cell. Post-translational modifications (PTM) of BER proteins offer an intriguing possibility to ensure that the components involved act at the right time at the chromatin in the nucleus (reviewed in Refs. [31,32]). PTM's likely involved in this regulation are phosphorylation, acetylation, sumoylation, mono- and polyubiquitylation as well as methylation (Table 1). Dianov and his group showed that BER components are targeted for destruction by the E3 ubiquitin ligase CHIP under normal conditions. However, when DNA damage occurs, those components undergo stabilization to increase the cellular capacity to perform BER [33]. In their work, Dianov and his group found that proteins such as pol β, XRCC1 and DNA ligase III are stabilized when they are bound to chromatin, forming an active repair complex. But when they are not attached to chromatin, meaning that they are not engaged in DNA repair, those components are polyubiquitinated by the concerted action of the E3 ubiquitin ligases CHIP and Mule, and consequently degraded. In a "preview" in the same issue of Mol. Cell Sobol concluded, that "the next goal is now to assess the crosstalk between PTM's, the ability to form productive repair complexes and the stability of these complexes" [34]. Here the concept was put forward that a single PTM, such as phosphorylation, might positively or negatively influence the enzyme's function and constitute a signal for further PTM's.

Initial data concerning the regulation of pol λ in vivo came from a study performed in our laboratory in 2005 when, in a proteomic search for novel interaction partners of pol λ by affinity chromatography, we found cyclin dependent kinase 2 (Cdk2) to interact with pol λ [35]. We showed that pol λ can be phosphorylated in vitro by several Cdk/cyclin complexes, including Cdk2/cyclin A, in its proline-serine rich domain (Fig. 1). Phosphorylation by Cdk2/cyclin A did not affect any biochemical properties of pol λ but the level of this PTM was decreased when pol λ interacted with PCNA, the ring-like moving platform that can interact with 10 different pols (reviewed in [2]). Finally, the phosphorylation-pattern of pol λ in vivo reflected the presence of Cdk2-cyclin A during the cell cycle. In a follow-up work, we could further demonstrate that pol λ was phosphorylated at four distinct sites, among which phosphorylation at Thr553 had a strongest impact on its stability [36].

## 3. Ubiquitylation of DNA polymerase λ and its implications in repair of 8-oxo-G

When we further investigated the impact of the Thr553 phosphorylation on the stability of pol λ, we found that an increase in the phosphorylation positively correlates with the levels of

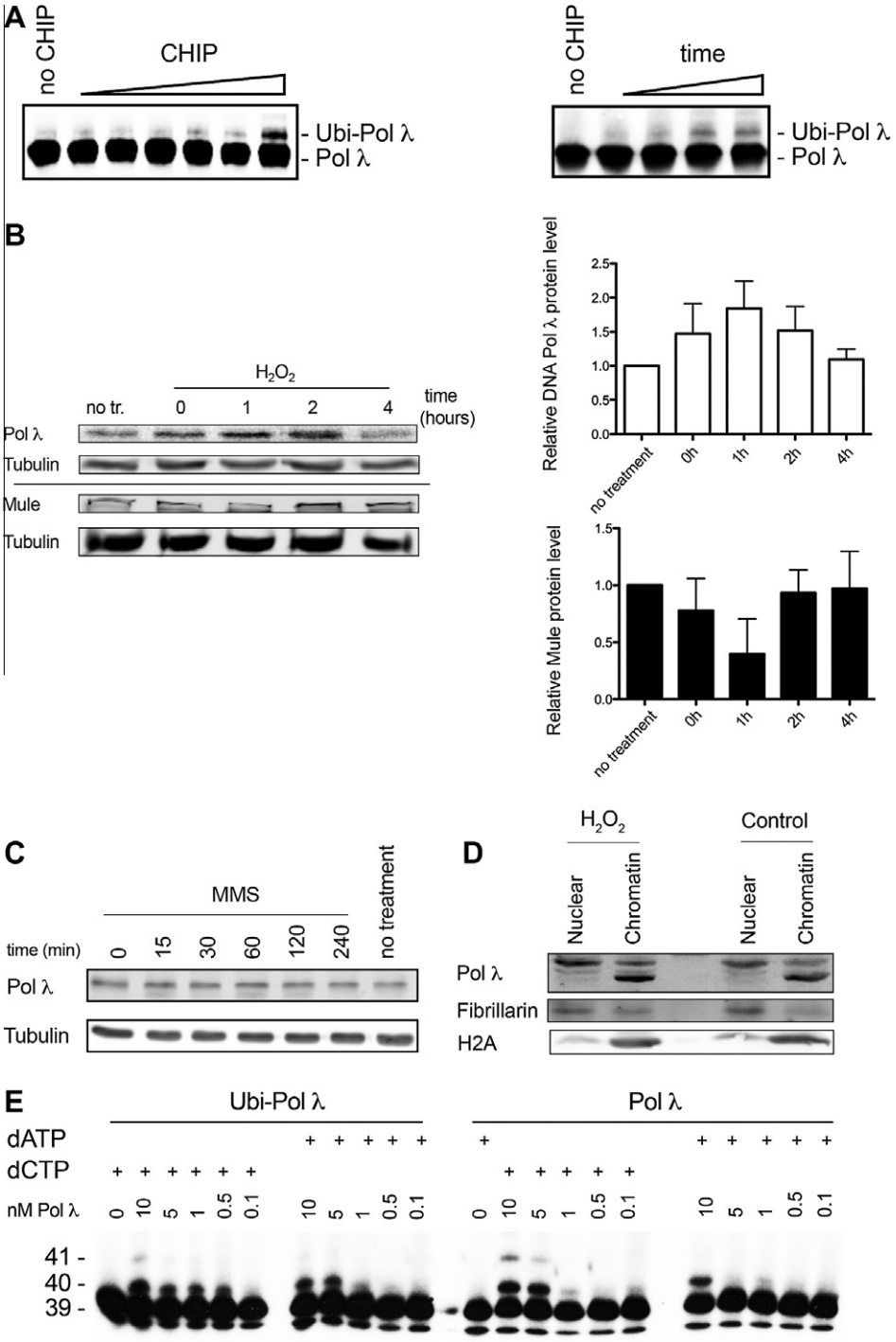


**Fig. 2.** Regulation of DNA polymerase λ by ubiquitination in response to oxidative DNA damage. (A) The CHIP E3 ligase ubiquitinates pol λ in vitro. The amounts of CHIP E3-ligase, titrated in the presence of E1 activating enzyme and the 10 E2 conjugating enzymes, were 25, 50, 100, 200, 500 and 1000 ng, with 200 ng of pol λ, respectively. (B) HeLa cells were treated for 1 h with $H_2O_2$ (1 mM) and the cells were subsequently released for the indicated time points. It can be seen that pol λ protein levels (white bars) in whole HeLa cell extracts increase, while the levels of the Mule E3 ligase (dark bars) decrease, with the effect being most prominent 1 h upon the release. Values presented on the right are mean of three independent experiments. Error bars are ±SD values. For description of the experiment see text. (C) When an analogous experiment in HeLa cells was performed upon treatment with MMS (500 μM) for 1 h, no changes in pol λ levels could be observed. The experiment was carried out as described in B. (D) Pol λ levels upon 1 h $H_2O_2$ (1 mM) treatment and 2 h release in HeLa cell nuclear and chromatin fractions, suggesting an increase in both of the fractions. (E) Functional consequences of ubiquitination on pol λ activity. The single nucleotide incorporation over an 8-oxo-G immediately following the primer terminus (so-called standing start conditions) were performed as outlined in Ref. [12]. Note that the in vitro monubiquitinated pol λ is less active in correct TLS over 8-oxo-G.

pol λ in the cell. This increase was found to be due to the fact that the phosphorylation of Thr553 protected pol λ from being ubiquitinated and subsequently degraded via the proteasomal pathway [36]. The phosphorylation-dependent stabilization was shown to take place in the late S and G2 phase, consistent with a possible role of pol λ in the post-replicative repair of A:8-oxo-G mispairs, likely enabling pol λ to properly conduct repair of damaged DNA during and after S phase [13]. It remains to be

investigated in more detail by what other means pol λ is regulated in vivo and how this regulation influences its activity.

To pursue our interest in the issue of shedding more light on the regulation of pol λ in the cell, we set out to determine which of the many cellular E3 ubiquitin ligases might be responsible for pol λ ubiquitination. Due to the fact that pol λ is closely related with pol β, we hypothesized that the ubiquitin E3 ligases that are involved in the ubiquitination of pol β might perform the task in the case of pol λ as well. For this end, we performed an in vitro ubiquitination assay, as originally developed by the Dianov's group [33].

In this assay, as for pol β [33], we found that pol λ can be ubiquitinated in vitro by the E3 ligase CHIP in dependence of the E1 activating enzyme and the 10 E2 conjugating enzymes (H2, H3, H5a, H5b, H5c, H6, H7, H8, H10 and H13) (Fig. 2A). Additionally, we found that pol λ can also be ubiquitinated by Mule in a similar in vitro reaction and that the extent of this reaction is concentration-dependent as well (data not shown).

8-oxo-G is a common lesion that arises from insults to DNA caused by reactive oxygen species (ROS) which in turn stem from various different endogenous and exogenous sources [37–40]. The steady-state levels of 8-oxo-G have been estimated to be around 1000-2000 lesions per cell per day in normal tissues and up to 100,000 per cell per day in the case of cancer [41,42]. 8-oxo-G is considered a mutagenic lesion as it readily pairs with an incorrect A instead of the correct C when present in *syn* conformation. This is due to the fact that an 8-oxo-G base pairing with its correct counterpart C induces template and polymerase distortions as they are seen when a pol encounters a mismatch [43]. Data obtained in our laboratory implicate pol λ to play a pivotal role in the post-replicative repair pathway of 8-oxo-G lesions by incorporating the *correct* nucleotide C opposite an 8-oxo-G lesion up to 1200 times more faithfully than any other pol tested [11–13].

In light of this situation, we expect the presence of pol λ to be particularly important during the late S and G2 phase of the cell cycle, where such a post-replicative repair step is needed. This is supported by the fact that pol λ is stabilised exactly during these cell cycle phases [36]. Additionally, the model would predict pol λ to be needed whenever there is oxidative DNA damage present that can give rise to 8-oxo-G lesions. A rise in the supply of pol λ could be achieved by either an increased production of the protein (by means of increased transcription and/or translation), or by a decrease in its turnover due to lower degradation via the ubiquitin-proteasome-system (UPS). Keeping the amount of pol λ constant, but stimulating its enzymatic activity would be an additional mean of coping with more 8-oxo-G damage under conditions of oxidative stress. To test whether an increase in oxidative DNA damage leads to a change in pol λ protein levels, we treated HeLa cells with hydrogen peroxide ($H_2O_2$) for the duration of 1 h, after which the cells were washed and released into normal medium again. A time-course analysis of whole cell extracts for pol λ protein levels revealed that pol λ levels increase up to 1 h after the treatment, followed by a phase in which the protein amount slowly decrease again to reach initial levels 4 h after the treatment (Fig. 2B). This finding demonstrates that cellular levels of pol λ seem to be responsive to the levels of oxidative DNA damage, in this case inflicted by the use of $H_2O_2$. As we had established that pol λ can be ubiquitinated in vitro by Mule, we were interested in monitoring whether this increase in pol λ could be due to a change in levels of Mule upon treatment. Indeed, when protein levels of Mule were analysed in the same experiment, it became clear that they responded reciprocally to the levels of pol λ, reaching the lowest levels 1 h after treatment (Fig. 2B). These data are in line with Mule regulating levels of pol λ in response to oxidative DNA damage. The next question that arose was whether pol λ levels respond similarly to any other kind of genotoxic insult or if the observed increase was a specific response to oxidative stress. Thus, we tested how the levels of pol λ respond to methyl methane sulfonate (MMS), an alkylating DNA damaging agent not known to induce 8-oxo-G in particular. In contrast to the treatment with $H_2O_2$, we could not observe any effect on the pol λ protein levels by MMS (Fig. 2C). This result indicated that an increase in pol λ levels is only induced when 8-oxo-G is produced by the presence of oxidative stress. When a cell fractionation experiment of HeLa cells was performed after 1 h of $H_2O_2$ treatment and subsequent release into normal medium for 2 h, we found an accumulation of pol λ protein in both the nuclear and chromatin-bound fractions (Fig. 2D). Thus, it seems that, after oxidative stress, also the fraction of the pol that is actively working on the DNA is increased and not only the 'backup-pool' present in the nucleus. This result is directly in line with the hypothesis that pol λ is up-regulated upon oxidative DNA damage to more efficiently perform its repair functions.

Finally, we were interested in looking at the functional consequences of ubiquitination on the enzymatic activity of pol λ. To test this, we used a single-nucleotide incorporation assay as originally published in [12]. This assay uses a standing start primer/template combination that allows the quantitative and qualitative monitoring of the incorporation step of either incorrect A or correct C opposite 8-oxo-G. The first incorporation step in this set-up is taking place opposite 8-oxo-G, while the second templating base is a normal G. Thus, when C is used in the reaction, two products can be observed: the first one representing the correct incorporation opposite 8-oxo-G and the second one an elongation step of one nucleotide. On the contrary, when A is used, only one incorporation event will take place which represents the erroneous incorporation of A opposite 8-oxo-G. Using this assay with 1 μM of the respective nucleotides, we found that in vitro ubiquitylation of pol λ strongly decreased the correct 8-oxo-G TLS activity of pol λ (Fig. 2E), hinting at the possibility that ubiquitination decreases the enzymatic activity of pol λ, additionally to targeting it for proteasomal degradation.

## 4. Summary and perspectives

Taken together, this assembly of very preliminary data suggests the existence of an inducible repair mechanism for correct incorporation of C opposite 8-oxo-G in mammalian cells upon induction of oxidative DNA damage. This induction might be controlled by the ubiquitin E3 ligases CHIP and/or Mule. It will be appealing to elaborate the possible role of ubiquitination in the regulation of pol λ. This is of particularly high interest as there is increasing evidence hinting towards the fact that misregulation of pol λ and also other TLS pols including pol β can lead to diseases in general [44] and to cancer in particular [45].

## 5. Conflict of interest

The authors declare no conflict of interest.

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## “Regulation of oxidative DNA damage repair by DNA Polymerase λ and MutYH by cross-talk of phosphorylation and ubiquitination”

**Enni Markkanen**, Barbara van Loon, Elena Ferrari, Jason L. Parsons, Grigory L. Dianov and Ulrich Hübscher

This manuscript, in which I am the first author, shows the findings regarding the interplay of phosphorylation and ubiquitination in the regulation of cellular Pol λ levels and its functional impact *in vivo*.

# Regulation of oxidative DNA damage repair by DNA polymerase λ and MutYH by cross-talk of phosphorylation and ubiquitination

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**It is of pivotal importance for genome stability that repair DNA polymerases (Pols), such as Pols λ and β, which all exhibit considerably reduced fidelity when replicating undamaged DNA, are tightly regulated, because their misregulation could lead to mutagenesis. Recently, we found that the correct repair of the abundant and highly miscoding oxidative DNA lesion 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxo-G) is performed by an accurate repair pathway that is coordinated by the MutY glycosylase homologue (MutYH) and Pol λ in vitro and in vivo. Pol λ is phosphorylated by Cdk2/cyclinA in late S and G2 phases of the cell cycle, promoting Pol λ stability by preventing it from being targeted for proteasomal degradation by ubiquitination. However, it has remained a mystery how the levels of Pol λ are controlled, how phosphorylation promotes its stability, and how the engagement of Pol λ in active repair complexes is coordinated. Here, we show that the E3 ligase Mule mediates the degradation of Pol λ and that the control of Pol λ levels by Mule has functional consequences for the ability of mammalian cells to deal with 8-oxo-G lesions. Furthermore, we demonstrate that phosphorylation of Pol λ by Cdk2/cyclinA counteracts its Mule-mediated degradation by promoting recruitment of Pol λ to chromatin into active 8-oxo-G repair complexes through an increase in Pol λ's affinity to chromatin-bound MutYH. Finally, MutYH appears to promote the stability of Pol λ by binding it to chromatin. In contrast, Pol λ not engaged in active repair on chromatin is subject for proteasomal degradation.**

base excision repair | Mule E3 ubiquitin ligase

Genetic stability is of crucial importance for any form of life and if not properly maintained can result in many human diseases (1). Reactive oxygen species (ROS) are among the many insults that can affect the stability of DNA by causing damage to the highly reactive DNA bases, such as guanine. Because of its prevalence and high mutagenic potential, 8-oxo-2′-deoxyguanine (8-oxo-G) is recognized as one of the most abundant mutagenic oxidative DNA lesions arising from such insults (reviewed in ref. 2). The cardinal problem with 8-oxo-G is that the majority of polymerases (Pols), including the three replicative Pols α, δ, and ϵ, bypass 8-oxo-G in an inaccurate manner by frequently incorporating the "wrong" adenine (A) opposite 8-oxo-G. This error can lead to the formation of GC → TA transversion mutations, which in turn can give rise to diseases such as cancer (3). In sharp contrast to the other Pols, mammalian Pol λ, a member of the X family Pols (4), is the main Pol capable of correctly handling an oxidatively damaged DNA strand with very high fidelity in collaboration with the auxiliary factors proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A), and incorporates over 1000-fold more efficiently the correct cytosine (C) opposite 8-oxo-G than the incorrect A (5, 6) in vitro. Furthermore, we have shown the existence of an accurate repair pathway for 8-oxo-G that is coordinated by the MutY glycosylase homologue (MutYH) and Pol λ in vitro and in vivo (7). These findings suggest that Pol λ is the most likely candidate among the fifteen mammalian Pols to play an important role in the accurate repair of oxidative DNA lesions and that this task is achieved by correctly using the damaged (oxidized) DNA strand as a template (7, 8).

Components of DNA repair complexes and especially of base excision repair (BER) need to be tightly regulated in order to guarantee that they are active only when needed [(9) and as discussed in ref. 10]. This regulation is of special importance for the DNA repair Pols, which show a much lower fidelity in polymerization of long stretches of DNA than the replicative Pols, and therefore could introduce many point mutations when replicating undamaged DNA. This hypothesis is supported by increasing evidence that deregulation of Pol λ and also other translesion synthesis Pols including Pol β can lead to diseases in general (11) and cancer in particular (12). Also, it has been shown that repair Pols are overexpressed in many tumors, a feature that may contribute to disease manifestation (13) further strengthening the idea that a tight control of repair Pols is pivotal. Nevertheless, the regulation of DNA repair enzymes, and Pols in particular, is so far poorly understood. Posttranslational modifications (PTMs) constitute a fascinating means of regulation to ensure proper temporal and spatial organization of repair components in the cell. Data from the Dianov lab have shown that BER components, such as Pol β, a close relative to Pol λ, undergo ubiquitination by the ubiquitin E3 ligase carboxy terminus of Hsc70 interacting protein (CHIP), which is an E3 ubiquitin ligase containing a C-terminal U box domain providing interaction with an E2 enzyme and an N-terminal tetratricopeptide mediating its interaction with heat shock proteins (14). CHIP plays an important role in the heat shock response (15, 16) and has been shown to be involved in regulating cellular levels of proteins like p53 (17). CHIP-mediated ubiquitination of Pol β leads to its degradation by the proteasome under normal circumstances (9, 18). However, upon DNA damage, those BER proteins are stabilized and recruited to chromatin to fulfill their roles in the maintenance of genomic integrity in vivo.

To date, very little is known about the regulation of Pol λ during the cell cycle. In previous work, we have shown that Pol λ interacts with cyclin-dependent kinase 2 (Cdk2) and is phosphorylated in vitro by the Cdk2/cyclinA complex (19). Phosphorylation per se does not affect the polymerization activity of Pol λ, but phosphorylation is decreased when Pol λ interacts with PCNA. Furthermore, the phosphorylation pattern of Pol λ coincides with the presence of Cdk2/cyclinA during the cell cycle. In follow-up

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work we demonstrated that phosphorylation prevents Pol λ from being degraded by the ubiquitin-proteasome pathway in vivo (20).

In the present study, we were interested in elucidating how the levels of Pol λ are controlled, how phosphorylation promotes its stability, and how the engagement of Pol λ in active repair complexes is coordinated. We found that Pol λ can be ubiquitinated by the E3 ligase Mule in vitro and in vivo and that this interaction is functionally connected to the phosphorylation-dependent stabilization of Pol λ by Cdk2/cyclinA. Importantly, the control of Pol λ levels by Mule has functional consequences for the ability of mammalian cells to deal with 8-oxo-G lesions in vitro. Furthermore, we demonstrate that phosphorylation of Pol λ by Cdk2/cyclinA counteracts its Mule-mediated degradation by promoting recruitment of Pol λ to chromatin into active 8-oxo-G repair complexes through an increase in Pol λ's affinity to chromatin-bound MutYH in vitro and in vivo. Finally, MutYH appears to promote the stability of Pol λ by binding it to chromatin. In contrast, Pol λ not engaged in active repair on chromatin is subject for proteasomal degradation. Our data elucidate how precisely and tightly PTMs can control Pol λ's activity status along with its overall cellular levels by orchestrating its subcellular localization and stability.

## Results

**Identification of Mule as E3 Ligase for DNA Polymerase λ.** In order to shed more light on the regulation of Pol λ in vivo, we set out to identify the E3 ubiquitin ligase responsible for the ubiquitination of Pol λ. To this end, we tested fractions originating from an assay established by the Dianov lab (9), where Pol λ ubiquitination activity was purified from HeLa whole cell extracts in a series of biochemical fractionations via chromatographic columns combined with an in vitro ubiquitination assay (Fig. S1*A*). The final Mono Q fractions D14–D10 clearly displayed Pol λ mono- and polyubiquitination activity of Pol λ (Fig. 1*A*), and they were sent for mass spectrometric analysis to identify the E3 ligase present in the fractions. The MS/MS data clearly identified Mule to be the major ubiquitin E3 ligase present in these fractions (18). In the same fractions, the E3 ligase CHIP was also identified and has been shown to ubiquitinate Pol λ in vitro (21). Mule is a 482 kDa protein in which the catalytically active homologous to the E6-AP carboxyl terminus (HECT) domain has been mapped to the C-terminal 370 amino acids (22) and has previously been shown to play a role in the regulation of another member of the Pol X family, Pol β (18). We thus used the recombinant truncated catalytically active HECT domain of Mule and confirmed that this protein mono- and polyubiquitinates Pol λ in a concentration-dependent manner in vitro (Fig. 1*B*). The ubiquitination reaction could be efficiently supported by any of the three E2 conjugating enzymes H5b, H5c, or H7 (Fig. S1*B*). When the band of in vitro ubiquitinated Pol λ was excised from a Coomassie stained SDS-PAGE gel (Fig. S1*C*) and subjected to MS/MS analysis, two lysines (K27 and K273) were identified to be ubiquitinated (Fig. S1*D*). In vitro ubiquitination assays were performed by using Pol λ with mutated K27, K273, or both residues using a mutant ubiquitin, not capable of forming polyubiquitin chains, in order to better quantitatively visualize the total ubiquitination. Although the single K273R mutant did not display any significantly reduced in vitro ubiquitination by Mule, both the single K27R and the double (K27R/K273R; named K2R) ubiquitination-deficient mutants of Pol λ showed 10 and 5 times reduced in vitro ubiquitination by Mule, respectively (Fig. S1*E*), suggesting that K27 is the major site of ubiquitination. The residual ubiquitination of the 2KR mutant Pol λ that was observed most probably stems from other K residues in the vicinity of K27 and K273 that are minor ubiquitination sites and can be ubiquitinated more strongly after the loss of the two main ubiquitination sites identified in the MS/MS approach.

Next, we addressed the question of whether Mule also regulates the protein levels of Pol λ in vivo. To test this, we knocked down Mule in HEK 293T cells by siRNA and found that this knockdown resulted in a significant increase in Pol λ protein levels ($p = 0.006$) (Fig. 1 *C* and *D*). The increase in Pol λ levels upon knockdown of Mule was less pronounced, but still significant ($p = 0.006$), in HeLa cells (Fig. S2 *A* and *B*), which are known to overexpress Mule protein (23). At the same time, the mRNA level of Pol λ remained constant (Fig. S2*E*), which suggests that transcription of the POLL gene was not affected by the knockdown of Mule. The effect of the Lipofectamine control was similar to controls using either nonspecific siRNA or siRNA against luciferase (Fig. S3 *A* and *B*).

Mule directly binds and ubiquitinates targets like p53 (22) and, thus, represses p53-mediated tumor suppressor functions leading to cell cycle arrest or aging (24). In response to DNA damage, the alternative reading frame of the INK4a/ARF locus (ARF) protein is induced, inhibits the activity of Mule, and thus leads to a stabilization of Mule targets as p53 (22) and Pol β (18). Importantly, ARF has not been shown to influence the levels of Mule but merely to inhibit its ubiquitin ligase activity (18, 22). To test whether this inhibitory effect of ARF on Mule affects Pol λ levels as well, we next knocked down ARF by siRNA. As expected, protein levels of Pol λ decreased upon knockdown of ARF in either HEK 293T (Fig. 1*E* and *F*) in HeLa cells (Fig. S2 *C*

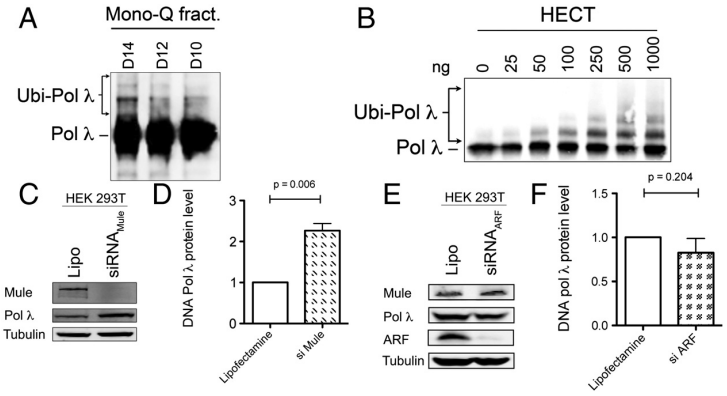


**Fig. 1.** Identification of Mule as an E3 ubiquitin ligase regulating cellular protein levels of DNA polymerase λ. (*A*) E3 ubiquitin ligase activity of the final Mono Q fractions from HeLa cell extracts (D14–D10) against Pol λ protein. (*B*) Ubiquitination of Pol λ by the purified recombinant HECT domain of Mule. (*C*) Effect of siRNA-mediated Mule knockdown on Pol λ levels in HEK 293T cells, analyzed by Western blotting. (*D* and *F*) Quantification of protein levels shown in *C* and *E* (three independent experiments each) showing mean + SD and *p* values obtained from one-sample *t* tests performed on the data. The Pol λ signal was normalized to tubulin. (*E*) Effect of siRNA-mediated ARF knockdown on Pol λ levels in HEK 293T cells, analyzed by Western blotting.

and *D*) and reflected the effect of increased Mule activity, even though the decrease was too small to prove significant ($p = 0.204$ for HEK and $p = 0.166$ for HeLa, respectively). At the same time, the mRNA level of Pol λ remained constant (Fig. S2*E*), which further supports the evidence that Mule regulates Pol λ protein levels by ubiquitination-dependent proteasomal degradation.

**The Correct 8-oxo-G Bypass by DNA Polymerase λ Is Regulated Through Mule.** We have previously shown that Pol λ is the Pol that bypasses 8-oxo-G containing DNA most accurately and incorporates the correct C opposite the lesion in vitro and that the specificity of correct nucleotide incorporation is provided by RP-A, PCNA, and MutYH in vitro (5–7). Therefore, we set out to determine whether modulation of Pol λ levels by Mule affects the amount of bypass of 8-oxo-G lesions. To assess this question, single-nucleotide incorporation assays using crude cell extracts with a primer-template combination that allows the quantitative monitoring of the incorporation of incorrect dATP or correct dCTP opposite a template containing 8-oxo-G (Fig. 2*A*) were performed as previously described (5). Crude cell extracts from HEK 293T cells treated with either siRNA against Mule [showing elevated levels of Pol λ (Fig. 1 *C* and *D*)] or Lipofectamine as a control were prepared, and their single-nucleotide incorporation activity opposite 8-oxo-G was analyzed. The extracts from cells with a Mule knockdown showed higher levels of correct C incorporation opposite 8-oxo-G than the control extracts, consistent with a role for Pol λ, or another Pol that is regulated by Mule, in the bypass of 8-oxo-G in vivo (Fig. 2 *B* and *C* and Fig. S4). When the relative differences of dCTP to dATP incorporation were analyzed, the extracts treated with siRNA against Mule showed a higher relative difference of dCTP to dATP incorporation (Fig. 2*C*) compared to the control-treated extracts. This difference could be further stimulated by the addition of PCNA and RP-A to the reaction, which is in line with our previous findings that PCNA and RP-A stimulate the correct bypass of 8-oxo-G by Pol λ (5).

To show that the amount of error-free bypass of 8-oxo-G lesions specifically depends on Pol λ and not on another Pol, and to confirm the functional effect of modulation of Pol λ levels by Mule in another cell line, we knocked down Mule by siRNA in Pol $\lambda +/+$ and Pol $\lambda -/-$ mouse embryonic fibroblasts (MEFs) (Fig. 2*D*). We found that in Pol $\lambda +/+$ MEFs the cellular protein levels of Pol λ were increased upon Mule knockdown (Fig. 2*D*). Importantly, only in cell extracts generated from MEFs containing Pol λ (Pol $\lambda +/+$ MEFs) was the incorporation of dCTP higher in extracts when Mule was knocked down in comparison to the Lipofectamine-only–treated cells (Fig. 2 *E* and *F*). This result corroborated the fact that the increase in 8-oxo-G lesion bypass observed after Mule knockdown specifically depends on Pol λ.

**The Phosphorylation Status of DNA Polymerase λ Regulates Its Subcellular Localization and thus Orchestrates Its Degradation Mediated by Mule.** In order to investigate the functional link between the stabilizing phosphorylation of Pol λ and its degradation by Mule, we analyzed HEK 293T cells stably transfected with either myc-Pol λ WT or myc-Pol λ 4A (a phosphorylation-deficient mutant of Pol λ lacking all four phosphorylation sites S167A, S177A, S230A, and T553A), because we previously observed that the lack of phosphorylation in this mutant increases its ubiquitination and thus leads to decreased cellular protein levels (20). As expected, examination of total cellular protein levels confirmed a reduction of 75% of the levels of Pol λ 4A compared to the WT protein (Fig. 3*A*). Furthermore, the majority of the Pol λ WT protein was present in a phosphorylated form (P-Pol λ), whereas the 4A mutant clearly showed a faster mobility following SDS-PAGE analysis. Treatment of these cells with siRNA against Mule dramatically increased the levels of Pol λ 4A by 3.9-fold compared to

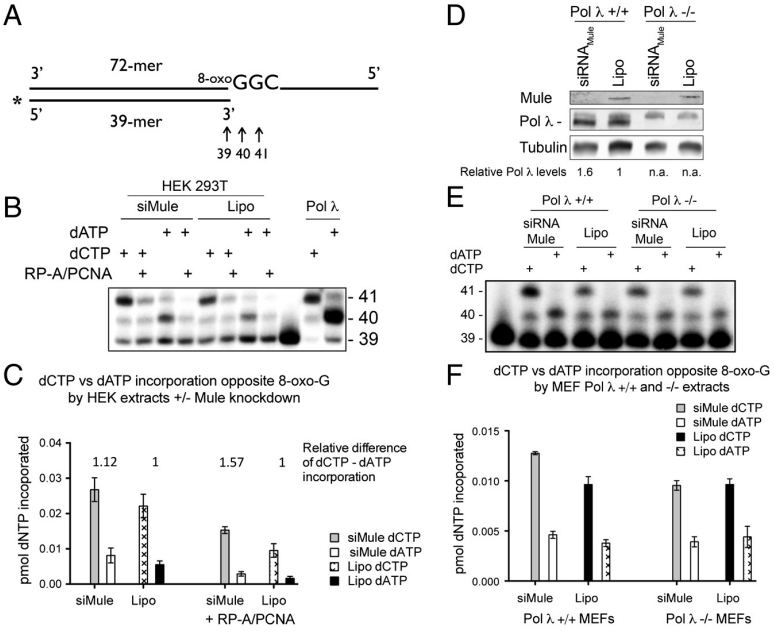


**Fig. 2.** The extent of error-free bypass of 8-oxo-G by DNA polymerase λ in human and mouse cell extracts is dependent on Mule. (*A*) 5′ labeled DNA primer/template pair used for the single-nucleotide incorporation assays. The first incorporation event is opposite 8-oxo-G. The incorporation of dATP yields a 40-mer product, whereas incorporation of dCTP gives rise to a 40-mer and a 41-mer. (*B*) Single-nucleotide incorporation by crude extracts from HEK 293T cells treated with siRNA against Mule. Experiments were performed with 10 μg of extracts, 10 μM dATP or dCTP, and +/− RP-A (40 nM) and PCNA (100 nM), respectively. (*C*) Quantification for *B*, mean of three independent experiments ±SD. (*D*) siRNA-mediated knockdown of Mule in Pol λ +/+ or −/− MEFs. (*E*) Single-nucleotide incorporation by extracts shown in *D*, performed with 10 μg of crude extracts and 0.5 μM dATP or dCTP, respectively. (*F*) Quantification for *B*, mean of four independent experiments ±SD.

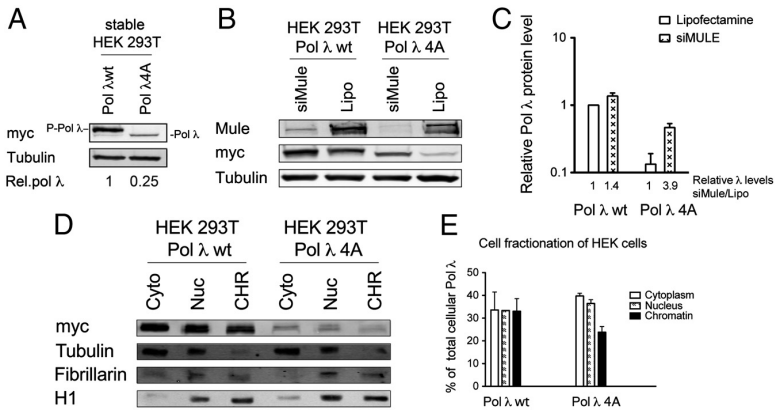


**Fig. 3.** Phosphorylation of DNA polymerase λ inhibits its ubiquitination by Mule and promotes its binding to chromatin. (*A*) Total cellular Pol λ protein levels in HEK cells stably transfected with myc-Pol λ WT or myc-Pol λ 4A constructs. Relative Pol λ levels were normalized to tubulin and are indicated below the respective column. P-Pol λ, phosphorylated form of Pol λ. (*B*) siRNA mediated knockdown of Mule or Lipofectamine control treatment in HEK cells stably transfected with myc-Pol λ WT or myc-Pol λ 4A constructs.(*C*) Quantification for *B*, mean of three independent experiments +SD. The Pol λ signal was normalized to tubulin. The relative Pol λ levels of siMule treated fractions to the respective Lipofectamine-treated controls are indicated. (*D*) Cell fractionation of HEK cells stably transfected with myc-Pol λ WT or myc-Pol λ 4A constructs. Cyto = cytoplasmic, Nuc = nuclear, and CHR = chromatin-bound fraction. (*E*) Quantification for *D*, mean of two independent experiments ±SD. The Pol λ signal was normalized to tubulin (for the cytoplasmatic fraction), to fibrillarin (for the nuclear fraction), or to histone H1 (for the chromatin-bound fraction). *H*1 = Histone 1.

an increase of 1.4-fold in the WT protein (Fig. 3 *B* and *C*), which hints toward an inhibitory effect of phosphorylation of Pol λ on its degradation by Mule. In order to examine the mechanism of this cross-talk between phosphorylation and ubiquitination more closely, we next fractionated the stable HEK 293T Pol λ WT and 4A cells into cytoplasmic, nuclear, and chromatin-bound protein fractions. Analysis of these extracts revealed a substantial decrease in chromatin-bound Pol λ 4A mutant compared to the WT protein (Fig. 3 *D* and *E*). A different fractionation protocol of these cells into soluble and chromatin fractions under harsher conditions further corroborated the difference in chromatin association between the WT Pol λ protein and its 4A mutant (Fig. S5). Taken together, these findings indicated that phosphorylation of Pol λ establishes or enhances its interaction either directly with chromatin or with a protein tightly bound to chromatin and that this binding to chromatin prevents Pol λ from being degraded by Mule-mediated ubiquitination.

**Phosphorylation of DNA Polymerase λ Enhances Its Interaction with MutYH on Chromatin and thus Regulates Its Activity State in the Cell.** The next question to be addressed was how and why phosphorylation orchestrates the subcellular localization of Pol λ and, more precisely, its binding to chromatin. We hypothesized that phosphorylation might enable and/or strengthen the interaction of Pol λ with a binding partner on chromatin. MutYH, a known interactor of Pol λ (7), recognizes 8-oxo-G:A mismatches and catalyzes the excision of the wrong A (reviewed in ref. 2). This step is followed by incision of the apurinic/apyrimidinic (AP) site by the action of apurinic/apyrimidinic endonuclease 1 (Ape1) to generate the substrate for Pol that performs the subsequent gap filling reaction (25). Thus MutYH is a protein that, according to our model of Pol λ's involvement in correct incorporation of C opposite 8-oxo-G (7), precedes the action of Pol λ in repair of 8-oxo-G lesions. To test whether MutYH influences the subcellular localization of Pol λ, we treated HEK 293T cells with siRNA against MutYH. Fractionation of siRNA- and Lipofectamine-only–treated cells revealed that both the total cellular levels as well as the chromatin-bound fraction of Pol λ were markedly reduced upon treatment with siRNA against MutYH (Fig. 4 *A* and *B*). This finding suggested that the interaction of Pol λ with MutYH stabilizes, and possibly also recruits, Pol λ to chromatin into active repair complexes and that this interaction is dependent on, or can be enhanced by, phosphorylation of Pol λ. Thus, we next asked whether the phosphorylation status of Pol λ has any effect on its binding to MutYH. To investigate this question, recombinant purified His-tagged Pol λ was phosphorylated by Cdk2/cyclinA in an in vitro phosphorylation assay and GST-pull-down experiments were carried out by using recombinant GST-MutYH and different amounts of His-Pol λ (Fig. 4*C*). The results of this interaction study clearly show that phosphorylation of Pol λ strongly enhances its interaction with MutYH.

**Chromatin-association of DNA Polymerase λ Is Inducible by Oxidative Stress.** The ultimate question to be answered was whether this fine-tuning of Pol λ levels had a physiological relevance under conditions of increased cellular stress due to oxidative DNA damage. To address this, cell fractionation experiments were performed by using T24 cells. T24 cells are human bladder carcinoma cells with the particular property that they are arrested in the G0 phase of the cell cycle by contact inhibition upon reaching 100% confluency. Importantly, these cells synchronously reenter the cycling phase upon seeding and, thus, enable analysis of synchronized cells without the need to use DNA damaging and cellular stress-inducing synchronization regimes, which could interfere with cell-cycle-dependent analysis of DNA repair pathways. G0-arrested T24 cells were seeded, either treated with 500 μM $H_2O_2$ for 45 min or mock-treated in the early S phase and fractionated at 2-h intervals upon release into normal medium. Analysis of chromatin fractions of $H_2O_2$-treated cells revealed an increase in chromatin-bound Pol λ levels that peaked to 1.7-fold 4 h after release compared to the control fractions, which did not show alterations in Pol λ levels (Fig. 4 *D* and *E*). Although the control cells clearly progressed through S to the G2/M phase, the $H_2O_2$-treated cells arrested in the S phase and failed to enter G2/M for the duration of the experiment (Fig. 4*D*). These results are consistent with an inducible chromatin recruitment of Pol λ dependent on oxidative DNA damage during the S phase.

## Discussion

The data presented in this paper shed more light on the intricate control mechanisms that are in place to regulate protein levels of Pol λ, its subcellular localization, and its engagement into active repair complexes on chromatin upon induction of oxidative DNA damage. We now identify Mule as an E3 ligase responsible for ubiquitination of Pol λ, leading to degradation of Pol λ via the

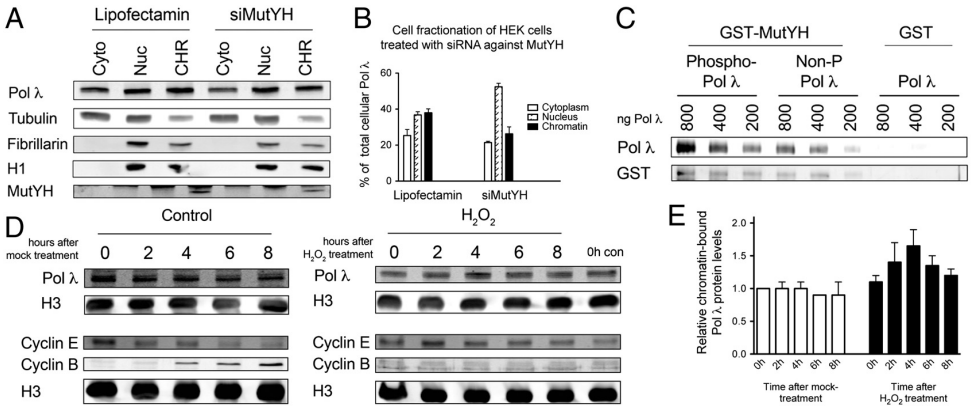


**Fig. 4.** Chromatin-binding of DNA polymerase λ is mediated by MutYH, enhanced by phosphorylation of DNA polymerase λ by Cdk2/cyclinA, and inducible by oxidative DNA damage. (*A*) Cell fractionation of HEK 293T cells treated with siRNA against MutYH. Cyto = cytoplasmic, Nuc = nuclear, and CHR = chromatin-bound fraction. (*B*) Quantification for *A*, mean of two independent experiments ±SD. The Pol λ signal was normalized to tubulin (for the cytoplasmic fraction), to fibrillarin (for the nuclear fraction), or to histone H1 (for the chromatin-bound fraction). *H1* = Histone 1. (*C*) GST-pull-down of different amounts of recombinant purified His-Pol λ by recombinant purified GST-MutYH after in vitro phosphorylation of Pol λ WT by Cdk2/cyclinA. Non-P-Pol, λ = non-phosphorylated control reactions for Pol λ, were carried out as the phosphorylation reaction but without the addition of Cdk2/cyclinA. Negative control for unspecific binding was carried out by adding GST instead of GST-MutYH to the reactions. Immunoblot: GST = GST-MutYH. (*D*) Analysis of chromatin-bound Pol λ levels in T24 cells upon induction of oxidative DNA damage in the early S phase by $H_2O_2$ treatment. The Pol λ signal was normalized to Histone H3 and the 0h control cell time point. The cell cycle progression was monitored by analysis of cyclin E and cyclin B. (*E*) Quantification of *D*, mean of two independent experiments ±SD.

ubiquitin-proteasome pathway. Although we found that Mule mainly monoubiquitinates Pol λ, the formation of di- or polyubiquitin chains can also be observed. It is still unclear whether it is Mule alone that is responsible for the degradation of Pol λ or if, like in the case of Pol β (18), monoubiquitination by Mule stimulates polyubiquitination by another E3 ligase. The possible role of the E3 ligase CHIP, also found to ubiquitinate Pol λ in vitro (21), in the regulation of Pol λ levels in vivo still remains to be assessed. In line with our findings concerning the regulation of Pol λ levels by Mule, we demonstrate that this regulation influences the capacity of HEK 293T cells to perform correct 8-oxo-G repair. Furthermore, we show that this repair is mainly carried out by Pol λ, as shown with single-nucleotide incorporation experiments using Pol $\lambda+/+$ or Pol $\lambda-/-$ MEF cell extracts depleted of Mule. Experiments assessing the phosphorylation of Pol λ, which has previously been found to stabilize Pol λ by decreasing its ubiquitination (20), reveal an decrease in chromatin association of nonphosphorylated Pol λ, as determined by fractionation experiments comparing HEK 293T cells stably transfected with Pol λ WT or Pol λ 4A phosphomutant. The phosphorylation-dependent chromatin binding protects and stabilizes Pol λ levels, as it prevents Pol λ from being shuttled to the cytoplasm, where it is subsequently ubiquitinated by Mule (a cytoplasmic protein) and degraded by the proteasome. Therefore, levels of Pol λ are controlled by means of changes in subcellular localization, which is dependent on the protein's phosphorylation status. MutYH is the DNA glycosylase that catalyzes the excision of an incorrect A opposite 8-oxo-G, followed by the action of Ape1, and thus precedes the role of Pol λ in incorporating a correct C opposite 8-oxo-G. We show that phosphorylation of Pol λ enhances its binding to MutYH and that depletion of MutYH in HEK 293T cells leads to a decrease in total cellular levels of Pol λ, as well as a decrease in the chromatin-associated Pol λ fraction. Phosphorylation of Pol λ by Cdk2/cyclinA has been shown to take place in the late S and G2 phases of the cell cycle. Considering that frequent misincorporation of A opposite 8-oxo-G is performed by the replicative Pols δ and ϵ, high levels of A:8-oxo-G mispairs are expected to be present immediately after DNA synthesis in the S phase. It is pivotal that those mispairs are corrected before mitosis proceeds, because otherwise GC to TA transversion mutations can manifest themselves. Under these circumstances, the Cdk2/cyclinA phosphorylation-dependent recruitment of Pol λ to chromatin makes a lot of sense, because Pol λ is so far the most likely candidate to work together with MutYH to achieve a correct repair of A:8-oxo-G lesions. This model is further substantiated by the finding that the chromatin-bound fraction of Pol λ can be increased 1.7-fold upon induction of oxidative stress (Fig. 4 *D* and *E*) in the early S phase and is in line with previous findings implicating the involvement of Pol λ in repair of oxidative DNA damage in vivo (7).

Studies assessing the regulation of Pol β protein levels have recently shown that the levels of Pol β are regulated by Mule and CHIP in vivo (9, 18). Importantly, these studies showed that ubiquitin-mediated proteasomal degradation mainly regulates the protein levels of Pol β and not its activity. So far, we do not have any evidence indicating that ubiquitination of either Pol λ or β influences the choice of a specific Pol in the BER pathway directly. Rather, the results from this study point to the possibility that this Pol choice is brought about by other PTMs, as phosphorylation, enabling a subtle regulation of Pol's subcellular localization, and thus contributes to the regulation of its degradation. It remains to be seen whether the polymerase activity of any of the repair Pols can be stimulated directly by means of PTMs, if PTMs control their association with other proteins to form entire repair complexes, or if their repair activity can be enhanced simply by changes in subcellular localization.

In the early Lindahl paper, a steady state of 100–500 8-oxo-G per cell was suggested (26). Subsequent work warned about artifactual DNA oxidation during isolation and suggested an amount of approximately 1,500 8-oxo-G per genome measured (see, e.g., ref. 27). Friedberg et al. indicated that around 1,000–2,000 8-oxo-G can be repaired per cell per day (28). Such a high steady-state level of DNA oxidation asks for a robust and tightly controlled repair system. With the data presented here, not only do we address the fine-tuning of Pol λ levels during physiological cellular conditions, we also provide evidence for what occurs upon induction of oxidative stress. ROS encountering a C:G base pair during any cell cycle phase devoid of DNA synthesis will lead to the formation of a C:8-oxo-G base pair, which is a substrate for Ogg1 (reviewed in ref. 2). Ogg1 will remove the damaged base 8-oxo-G,

and subsequent BER will take care of the resulting AP site. Consequently, 8-oxo-G lesions caused by ROS inflicted on the cell in any of these nonreplicative phases will mainly necessitate the action of Ogg1. On the other hand, A:8-oxo-G mispairs are thought to mainly arise from inaccurate bypass of an 8-oxo-G lesion by replicative Pols during the S phase. For this reason, late S and G2 are the phases during which the removal of A opposite 8-oxo-G is needed. This notion is supported by the fact that MutYH levels reach their maximum during the S phase (29) and that the repair of A:8-oxo-G mismatches in vivo by MutYH is fourteenfold more efficient when the substrate is replication-proficient compared to a nonreplicating one (30). Those results are in accordance with a replication-associated activity of MutYH. Hence, because Pol λ is stabilized by phosphorylation by Cdk2/cyclinA in late S and G2 as well (20), we believe that the stabilization of an interaction between MutYH and Pol λ during exactly these phases of the cell cycle can promote repair of A:8-oxo-G mismatches.

Taken together, we have unveiled an important feature in the dynamics and control of Pol λ, a repair Pol pivotal for correct repair of oxidative DNA damage, which is crucial for the maintenance of genetic stability. Our results are consistent with a model in which Pol λ is recruited to and/or retained on chromatin in a phosphorylation-dependent manner into active repair complexes by MutYH in the late S and G2 phases of the cell cycle. Importantly, this recruitment is increased upon exposure of S-phase cells to oxidative DNA damaging agents. This phosphorylation-dependent chromatin recruitment further protects Pol λ from being sent to the cytoplasm, where it undergoes ubiquitination by Mule and is then sent for proteasomal degradation.

## Materials and Methods

**In Vitro Ubiquitination of Pol λ.** Purification of the ubiquitination activity for Pol λ from HeLa whole cell extracts and the in vitro ubiquitination assays were performed as described in ref. 18.

**In Vitro Phosphorylation of Pol λ.** This experiment was performed as outlined in ref. 20.

**GST Pull-down Assay of Pol λ with MutYH.** This interaction was done as recently described in ref. 7. Single-nucleotide incorporation assays were performed as described in ref. 5 with modifications as indicated in the figure legends.

**RNAi Interference.** Cells were transfected by using the Lipofectamine RNAi max (Invitrogen) according to the manufacturer's protocol and analyzed 72 h after transfection.

**Statistical Analysis.** For all the statistical analysis, the program GraphPad Prism (www.graphpad.com) was used.

Full Materials and Methods can be found in *SI Materials and Methods*.

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## "*In vitro* gap directed translesion DNA synthesis of an abasic site involving human DNA Polymerases ε, λ and β"

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For this manuscript, I prepared the cell extracts that were used to provide the *in vivo* relevance to the biochemical data.

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# *In Vitro* Gap-directed Translesion DNA Synthesis of an Abasic Site Involving Human DNA Polymerases ϵ, λ, and β*[S]

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**DNA polymerase (pol) ϵ is thought to be the leading strand replicase in eukaryotes, whereas pols λ and β are believed to be mainly involved in re-synthesis steps of DNA repair. DNA elongation by the human pol ϵ is halted by an abasic site (apurinic/apyrimidinic (AP) site). In this study, we present *in vitro* evidence that human pols λ, β, and η can perform translesion synthesis (TLS) of an AP site in the presence of pol ϵ, likely by initiating the 3′OHs created at the lesion by the arrested pol ϵ. However, in the case of pols λ and β, this TLS requires the presence of a DNA gap downstream from the product synthesized by the pol ϵ, and the optimal gap for efficient TLS is different for the two polymerases. The presence of gaps did not affect the TLS capacity of human pol η. Characterization of the reaction products showed that pol β inserted dAMP opposite the AP site, whereas gap filling synthesis by pol λ resulted in single or double deletions opposite the lesion. The synthesis up to the AP site by pol ϵ and the subsequent TLS by pols λ and β are not influenced by human processivity factor proliferating cell nuclear antigen and human single-stranded DNA-binding protein replication protein A. The bypass capacity of pol λ at the AP site is greatly reduced when a truncated form of the enzyme, which has lost the BRCA1 C-terminal and proline-rich domains, is used. Collectively, our *in vitro* results support the existence of a mechanism of gap-directed TLS at an AP site involving a switch between the replicative pol ϵ and the repair pols λ and β.**

Chromosomal DNA replication in eukaryotic cells requires three DNA polymerases (pols)[3]: pol α, pol δ, and pol ϵ. pol α is the only polymerase that has an associated activity for synthesis of RNA primers and is able to extend from such primers by synthesizing short stretches of DNA (1, 2). Subsequently, processive DNA synthesis is resumed by pol δ and/or pol ϵ. Recent work in yeast supports a model wherein, during normal DNA replication, pol ϵ is primarily responsible for copying the leading strand, and pol δ is primarily responsible for copying the lagging strand (3).

Abasic sites (AP sites) arise frequently by spontaneous hydrolysis of purines in DNA, represent a common intermediate of numerous DNA repair systems, and are among the most common endogenous DNA lesions generated during normal cell growth (4, 5).

An AP site poses a serious problem to the advancement of a pol because the modified base has lost its coding capacity. Accordingly, its replication requires the intervention of one or more Y family polymerases in a process called translesion synthesis or TLS (for reviews see Refs. 6, 7). Recent publications have shown that among these polymerases human pol η was able to insert nucleotides opposite the AP site and extend primers further past the lesion *in vitro* (8). Moreover, pol η showed higher abasic lesion bypass capacity *in vivo* than pols ι, κ, and Rev1 (9). Furthermore, it has also been reported that an AP site could be bypassed *in vitro* by polymerases of other families such as pol α (10), pol δ in the presence of the processivity factor PCNA (11), and pols λ and β (12). Concerning pol ϵ, a limited capacity of TLS of an AP site has been reported for the yeast enzyme (13) but not for its human counterpart, which appeared to be blocked mainly at the base preceding the lesion with minor incorporation opposite to it (14).

A widely accepted model of DNA lesion bypass is the polymerase-switching model that is believed to act at the replication fork to enable replication to continue by bypassing DNA lesions that halt the progression of the replicative polymerases. In this model, protein-protein interactions mediate a pol handoff at the template-primer terminus from the replicative pol to one or more specialized polymerases. In eukaryotes, this switching appears to be mediated by a monoubiquitinated form of the processivity clamp factor PCNA. A further switch restores the replicative pol to the primer terminus, and accurate synthesis resumes (reviewed in Ref. 7).

Conversely, a second model, named gap-filling model, can be envisaged to account for TLS-assisted bypass of DNA lesions outside the context of the replication fork, and its purpose

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[3] The abbreviations used are: pol, polymerase; TLS, translesion synthesis; PCNA, proliferating cell nuclear antigen; BRCT, BRCA1 C-terminal domain; NER, nucleotide excision repair; LP-BER, long patch base excision repair; AP, apurinic/apyrimidinic site; RPA, replication protein A.

would be to seal gaps containing lesions resulting from re-priming events or processing of closely spaced lesions on opposite DNA strands (7). In contrast to the polymerase-switching model, the molecular mechanism(s) underlying the gap-filling model are still largely unknown.

In this work, we report *in vitro* DNA gap-dependent TLS at an AP site by human DNA repair polymerases, pol λ and pol β, in the presence of the replicative human pol ϵ. Human pol η can also perform TLS that does not appear to depend on DNA gaps. We also present evidence that TLS by pols λ and β is not influenced by the human processivity factor PCNA and the human single-stranded DNA-binding protein RPA. We also show that the capacity of pol λ to bypass an AP site is greatly reduced when a truncated form of the enzyme, which has lost the BRCT and proline-rich domains, is used. Taken together, our *in vitro* results may suggest the existence of a novel pathway of DNA repair, gap-directed TLS involving human pols ϵ, λ, and β.

## EXPERIMENTAL PROCEDURES

*Proteins*—Recombinant human pol λ, RPA, and PCNA were expressed and purified as described previously (15–17). Recombinant pol λ(244–575) mutant was expressed as described previously (18) and purified as described previously (19). Recombinant human pol β was from Trevigen Inc. (Gaithersburg, MD). Recombinant human pol η was from Enzymax (Lexington, KY). Human pol ϵ was purified from HeLa cells through six purification steps as described previously (14, 20). The glycerol gradient fraction used in this study had a specific activity of 24,000 units/mg. Its purity was estimated to be >50%, and the fraction was devoid of other replicative polymerases (14).

*DNA Substrates and Chemicals*—The 100-mer oligonucleotide templates, either undamaged or containing a synthetic AP site (tetradroxyfuran moiety), and the oligonucleotide primers were from Eurogentec. The oligonucleotides complementary to the 5′ end of the templates were from Sigma, and all molecules carried a 5′-phosphate. All oligonucleotides were purified by PAGE. The DNA substrates used in this study are indicated in Table 1. Primers were 5′-labeled with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-$^{32}$P]ATP according to the manufacturer's protocol. Each primer was mixed with the templates at an equimolar ratio. When necessary, the oligonucleotides complementary to the 5′ end of templates were added to the reaction at an oligonucleotide/template ratio of 2:1 to ensure complete hybridization to all templates. [γ-$^{32}$P]ATP was from PerkinElmer Life Sciences; dNTPs were from Fermentas Life Science, and ddGTP was from GE Healthcare. 20× glycerol tolerant gel (GTG) buffer was from United States Biochemical Corp.

*Primer Extension Assays*—Reaction solutions of 10 μl were incubated at 37 °C and contained 0.15 pmol of DNA templates, 50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 200 μg/ml BSA, and 100 μM each of dATP, dCTP, dGTP, and dTTP. The incubation times and the amount of proteins used are indicated in the legends of the figures. The reactions were stopped by adding 5 μl of stop solution containing 0.1% xylene cyanol and 0.1% bromphenol blue in 90% formamide. Before loading onto the gel, samples were denatured by heating at 100 °C for 3 min. The reaction products were resolved on denaturing PAGE (7 M urea, 10% acrylamide) run in GTG buffer (90 mM Tris, pH 9, 30 mM taurine, and 5 mM EDTA) and visualized and quantified using phosphorimager (GE Healthcare) and ImageQuant software. The percentage of TLS was calculated as the ratio of the intensity of bands present at the position opposite the lesion or beyond to the intensity of these bands plus the intensity of the band present one nucleotide before the lesion.

*DNA Sequencing of Reaction Products*—Reaction solutions of 10 μl were incubated at 37 °C and contained 50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 200 μg/ml BSA; 100 μM each of dATP, dCTP, dGTP, and dTTP; and 0.15 pmol of DNA templates containing a single AP site within either a 2- or a 4-nucleotide gap. The 2- and 4-nucleotide gap templates were replicated by pol ϵ for 15 min followed by addition of pol β or pol λ for 5 min, respectively. Gap-filled products were converted to 100-mer by addition of 10 units of T4 DNA ligase and incubation at 37 °C for 10 min. Ligated products were amplified by 30 cycles of PCR in the presence of 1.25 units of Pfu DNA polymerase (Fermentas) and 25 pmol of both 5′-ACTACATTTACTTTCAATTACATAATTTCAAATCCTAATAATCT-3′ and 5′-TAAGGTAGTAGTATTATAAATTATG-3′ primers. PCR-amplified products from three independent reactions were pooled and purified. 29 and 28 individual clones were sequenced after TOPO cloning of the purified products for pols β and λ, respectively (Millegen, Toulouse, France).

*Gel Mobility Shift Assay*—Reaction of 10 μl contained 0.3 pmol of DNA substrates and 1.5 pmol of pol β in 50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 200 μg/ml BSA. After an incubation time of 15 min at 37 °C, 1 μl of loading buffer (50% glycerol, 0.20% bromphenol blue, 0.20% xylene cyanol, and 0.2 M EDTA) was added, and the mixture was loaded on an 8% native gel in 0.5× TBE running buffer (89 mM Tris borate, pH 8.3, and 2 mM EDTA) and electrophoresed at 4 °C at 10 V/cm for 3 h.

## RESULTS

*DNA Polymerase λ Requires a DNA Gap with a Specific Length to Perform Translesion Synthesis in the Presence of DNA Polymerase ϵ*—DNA elongation by human pol ϵ is severely blocked by an abasic site (14), although human pol λ can bypass such a DNA lesion (12). We therefore investigated whether pol λ could resume DNA synthesis when pol ϵ was stalled at an AP site. For initial experiments, the 100-mer template shown in Table 1a was used. The template contains a unique synthetic AP site at a defined position, and it was annealed to a primer of 44-mer, because a minimal primer length of ≈40 bp is required to maximize the binding and processivity of pol ϵ (21). As reported previously (14), when incubated with the 100- to 44-mer template-primer (which is defined in this work as single-stranded template-primer), pol ϵ was unable to replicate past the abasic site and stopped primarily at the base preceding the lesion, with some incorporation opposite the lesion (*lane 2* of Fig. 1*A* and quantified in Fig. 1*B*). Addition of pol λ (0.25 pmol) at this stage and further incubation for 5 min did not resume DNA synthesis (Fig. 1*A*, *lane 4*).

pol λ is a family X pol that is involved in DNA repair and has higher incorporation efficiency on gapped than single-stranded

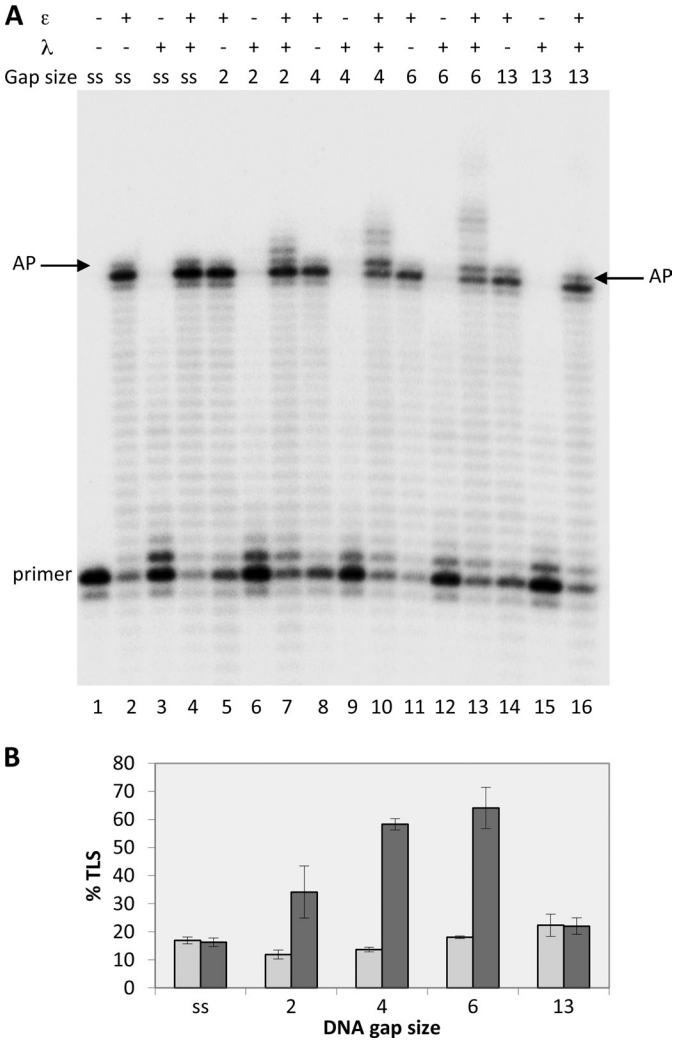


FIGURE 1. **Ability of DNA pol λ to perform translesion synthesis of an AP site in the presence of DNA pol ϵ depends on the gap size.** Experiments were performed with templates shown in Table 1, part a. The enzymes and the DNA substrates used are indicated at the *top*. *ss* (single-stranded) stands for template-primer with no oligonucleotides hybridized downstream from the AP site; *2, 4, 6,* and *13* indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A, lane 1,* no polymerase present. *Lanes 2, 5, 8, 11,* and *14,* reactions incubated for 35 min with 0.025 pmol of pol ϵ. *Lanes 3, 6, 9, 12,* and *15,* reactions incubated for 5 min with 0.25 pmol of pol λ. *Lanes 4, 7, 10, 13,* and *16,* reactions were incubated with 0.025 pmol of pol ϵ for 30 min; then 0.25 pmol of pol λ were added, and incubation was continued for 5 min. The positions of the primer and of the AP site are indicated. *B,* quantification of the percentage of TLS calculated as described under "Experimental Procedures." Mean ± S.D. values for three independent experiments are indicated. *Light gray bars,* pol ϵ alone. *Dark gray bars,* pols ϵ and λ together.

DNA (see Ref. 22 and references therein). We therefore constructed template-primers containing gaps of different lengths around the abasic site and tested the capacity of pol λ to perform TLS in presence of pol ϵ. To this purpose, we hybridized the 100:44-mer single-stranded template-primer with oligonucleotides of different lengths placed downstream from the abasic site. With these template-primers, arrest of the elongation catalyzed by pol ϵ at the AP site results in gaps of a size from 1 to 13 nucleotides *starting* from the base that follow the abasic site (Fig. 1*A*). Because pol λ possesses a 5′-deoxyribose-phosphate lyase activity and it has been shown that a 5′-phosphate present in a gap strengthens the binding of the enzyme (23), all oligonucleotides placed downstream from the AP site were synthesized with a 5′-phosphate.

As can be seen in Fig. 1*A*, DNA gaps of 2, 4, and 6 nucleotides starting from the lesion radically changed the behavior of pol λ that now acquired the capacity to perform TLS of the AP site in the presence of pol ϵ (see *lanes 7, 10,* and *13*). The gaps enabled pol λ to catalyze both incorporation opposite the lesion and beyond and to fill the gaps with no or little strand displacement synthesis consistent with the known limited strand displacement capacity of the enzyme (Fig. 2) (19). The TLS capacity of pol λ appears to increase from gaps of 2 nucleotides to gaps of 4–6, but it is dramatically reduced with a gap of 13 nucleotides (see quantifications in Fig. 1*B*), where the enzyme almost behaved as with the single-stranded template-primer in *lane 4*. Thus it appears that the TLS capacity of pol λ is strongly modulated by the size of the DNA gap.

As seen in Fig. 1, when 0.25 pmol of pol λ were incubated for 5 min with the substrates in the *absence* of pol ϵ, the products synthesized were all too short to reach the lesion (*lanes 3, 6, 9, 12,* and *15*). This finding suggested that pol λ catalyzed TLS by using the 3′OHs created by pol ϵ, arrested either at the base preceding the lesion or opposite it, rather than using shorter primers generated during its own synthesis.

To further clarify this point, an experiment was devised in which the template-primers shown in Table 1b were used. If one compares template 1a to 1b in Table 1, it can be seen that in the latter the sequence between the 3′OH of the primer and the AP site has been changed, so that the only cytosines present in the template are now within this sequence. This allows specific inhibition of any elongation from 3′OHs in this region when the chain elongation inhibitor ddGTP is used. Oligonucleotides were hybridized to this template-primer to create gaps of 4 and 13 nucleotides. The rationale of this approach is that *simultaneous* addition of both pol λ and ddGTP in the presence of the stalled pol ϵ will abolish any priming contribution *not* starting from a pre-existing 3′OH. The result of this experiment is shown in supplemental Fig. 1. Note that to maximize the inhibitory effect of ddGTP, a concentration of 1 pmol of pol λ instead of the usual 0.25 pmol was used. As expected, incubation of 1 pmol of pol λ for 5 and 10 min in the absence of pol ϵ resulted in increased synthesis compared with that seen previously with 0.25 pmol (compare *lanes 3* and *4* of supplemental Fig. 1 with *lane 3* of Fig. 1). Samples were incubated for 5 and 10 min, and at 10 min one can see some TLS because of the intrinsic AP bypass activity of pol λ. Addition of ddGTP restrained incorporation to the first G following the 3′OH of the primer (supplemental Fig. 1, *lanes 5* and *6*). On single-stranded template-primer, addition of 1 pmol of pol λ in the presence of pol ϵ induced some TLS, particularly at 10 min, and this is seen also for the template-primer containing the 13-nucleotide gap (supplemental Fig. 1, *lanes 7* and *8* and *15* and *16*). However, addition of ddGTP before addition of pol λ completely abolished this TLS, indicating that it was due to elongation by pol λ of pre-existing primers and not of those created by pol ϵ (supplemental Fig. 1, *lanes 9, 10* and *17, 18*). Interestingly, the situation appeared different with the template-primer containing the 4-nucleotide gap, where a substantial part of the TLS by a high

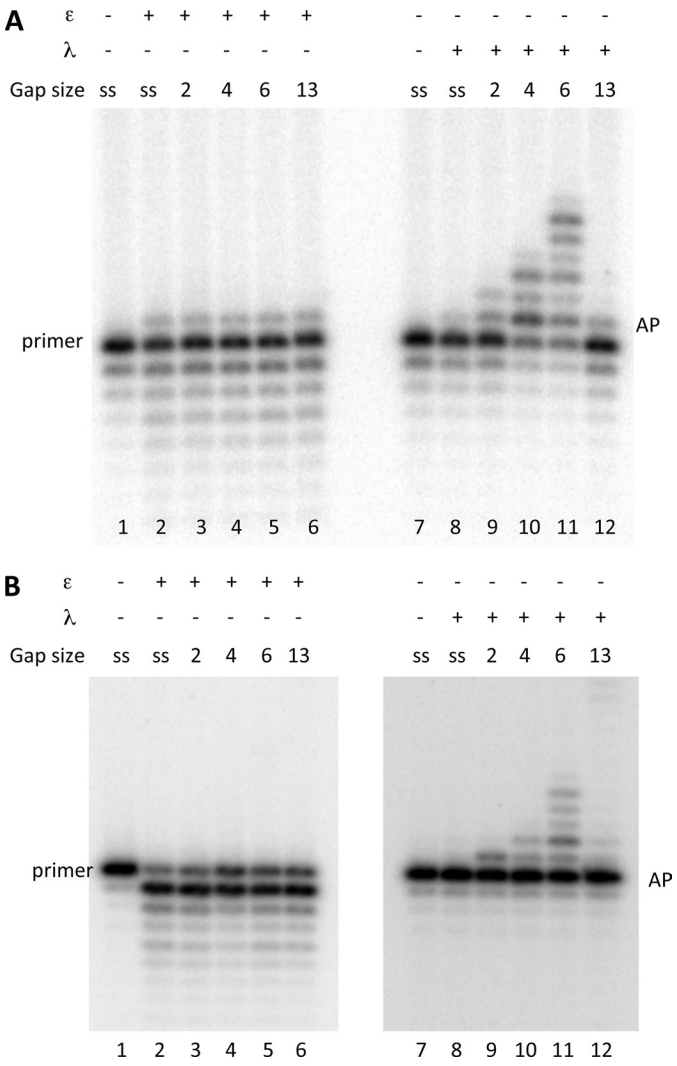


FIGURE 2. **DNA pol λ can extend both from a nucleotide preceding the AP site and from an A incorporated opposite to it when placed within a DNA gap, although DNA pol ϵ cannot.** Experiments were performed with the templates shown in Table 1c. The enzymes and the DNA substrates used are indicated at the *top*. *ss* stands for a template-primer with no oligonucleotides hybridized downstream from the AP site, and *2, 4, 6,* and *13* indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A,* experiments conducted with the template primed with the 45-mer terminating one nucleotide before the AP site. *Lane 1,* no polymerase present. *Lanes 2–6,* reactions incubated for 35 min with 0.025 pmol of pol ϵ. *Lane 7,* no polymerase present. *Lanes 8–12,* reactions incubated for 5 min with 0.25 pmol of pol λ. The positions of the primer and of the AP site are indicated. *B,* experiments conducted with the template primed with the 45-mer terminating with an *A* opposite to the AP site. *Lane 1,* no polymerase present. *Lanes 2–6,* reactions incubated for 35 min with 0.025 pmol of pol ϵ. *Lane 7,* no polymerase present. *Lanes 8–12,* reactions incubated for 5 min with 0.25 pmol of pol λ. The positions of the primer and of the AP site are indicated.

amount of pol λ took place *also* in the presence of ddGTP (supplemental Fig. 1, compare *lanes 11* and *12* with *13* and *14*), indicating that in this case pol λ could effectively use 3′OHs created at the lesion by pol ϵ.

To directly confirm that pol λ could extend past the lesion in presence of a gap starting from 3′OH generated by pol ϵ, as suggested by the experiments shown in Fig. 1 and supplemental Fig. 1, we created two template-primers in which the primer is either a 45-mer bearing a 3′OH at the base preceding the AP site or a 45-mer bearing the 3′OH at an A placed opposite the lesion (Table 1, template-primers 1c). The choice of the latter template-primer was motivated by our previous results that showed that either A or C is incorporated by pol ϵ opposite the AP site (14). To these template-primers, appropriate oligonucleotides were hybridized to generate gaps of 2, 4, 6, and 13 nucleotides, and they were used in reactions with 0.025 pmol of pol ϵ and 0.25 pmol of pol λ.

Fig. 2*A* shows the results of reactions using the primer ending at the nucleotide *preceding* the AP site. As expected, pol ϵ can incorporate in front of the lesion but is unable to elongate past it with all the substrates tested (*lanes 2–6*). On the contrary, pol λ alone can replicate past the AP site when gaps are present and, importantly, with a gap preference similar to that observed when the 3′OHs were generated by the arrest of pol ϵ at the lesion (compare *lanes 9–12* of Fig. 2*A* with *lanes, 7, 10, 13,* and *16* of Fig. 1*A*).

Fig. 2*B* shows the results of reactions with the primer bearing an A *opposite* the AP site. As can be seen, pol ϵ is unable to elongate from this nucleotide, likely because its 3′-5-′-exonuclease continuously excised the A, as indicated by the increased intensity of the band preceding the lesion (compare *lanes 2–6* of Fig. 2*A* with *lanes 2–6* of *B*). Unlike pol ϵ, pol λ can also replicate from the A opposite the AP site, again with a gap preference similar to the one observed in Fig. 1. It should be noted that, as shown in Fig. 1, the amount of λ used in our study essentially fills the gaps during TLS with no significant strand displacement synthesis.

Taken together these data strongly suggest that the presence of gaps of defined size is a major determinant allowing pol λ to substitute for pol ϵ to bypass the AP site (see also under "Discussion"). Related to our finding, it is interesting to note that a recent work has shown that the polymerization activity of human pol λ increases with DNA gaps from 1 to 4, remains constant for gaps from 4 to 7, and then drops for gaps from 7 to 10 nucleotides (22).

*Full-length DNA Polymerase λ Is Required for the AP Site Translesion Synthesis in the Presence of DNA Polymerase ϵ*—pol λ has two nonenzymatic domains at its N terminus, a BRCA1 C-terminal (BRCT) domain and a proline-rich domain (for review see Ref. 23). Little is known about the functions of these domains, but BRCT domains are known to mediate protein-protein and protein-DNA interactions (24), and both domains have been suggested to up-regulate or down-regulate fidelity of pol λ during gap filling activity, depending on the length of the gaps (22, 25). We therefore compared the AP site TLS capacities of pol λ WT and of the mutant form missing the BRCT and proline-rich domains (pol λ(244–575)) in the presence or absence of pol ϵ. The results of this comparison are shown in Figs. 3 and 4. Fig. 3*A*, quantified in *B*, shows that the λ(244–575), with both the 4- and 6-nucleotide gaps depicted in Table 1, had a severely reduced capacity to perform AP site translesion synthesis in the presence of pol ϵ compared with the WT (compare *lanes 5* with *6* and *lanes 7* with *8*). Note that the two enzymes displayed the same activity on single-stranded template-primer (*lanes 3* and *4* of Fig. 3*A*). Next, we examined the capacity of the two forms of pol λ to replicate past the AP site when initiating elongation from a 3′OH preceding the lesion, using the 100/45 template-primer shown in Table 1c. As can be

**TABLE 1**

**DNA templates**

**X** indicates the synthetic abasic site on the damaged template or a guanine on the undamaged template. All the oligonucleotides complementary to the 5′ end of the 100-mer templates bear a 5-phosphate.

a)

```
                                  1-13 gap
3'ATTCCATCATCATAATATTT ◄──────►
3'ATTCCATCATCATAATATTTAAT
3'ATTCCATCATCATAATATTTAATAC
3'ATTCCATCATCATAATATTTAATACC
3'ATTCCATCATCATAATATTTAATACCT
3'ATTCCATCATCATAATATTTAATACCTC
3'ATTCCATCATCATAATATTTAATACCTCC
3'ATTCCATCATCATAATATTTAATACCTCCA
3'ATTCCATCATCATAATATTTAATACCTCCAA
3'ATTCCATCATCATAATATTTAATACCTCCAAA                          TCTAATAATCCTAAACTTTAATACATTAACTTTCATTTACATCA5'
5'TAAGGTAGTAGTATTATAAATTATGGAGGTTTTXAGTGGGAAATAAAATATAGTAAAGATTATTAGGATTTGAAATTATGTAATTGAAAGTAAATGTAGT3'
```

b)

```
3'ATTCCATCATCATAATATTT
3'ATTCCATCATCATAATATTTAATACCTCC                             TCTAATAATCCTAAACTTTAATACATTAACTTTCATTTACATCA5'
5'TAAGGTAGTAGTATTATAAATTATGGAGGTTTTXACTCCCAAATAAAATATACTCCAGATTATTAGGATTTGAAATTATGTAATTGAAAGTAAATGTAGT3'
```

c)

```
3'ATTCCATCATCATAATATTT
3'ATTCCATCATCATAATATTTAATACCT
3'ATTCCATCATCATAATATTTAATACCTCC      TCACCCTTTATTTTATATCATTTCTAATAATCCTAAACTTTAATA5'
3'ATTCCATCATCATAATATTTAATACCTCCAA  ATCACCCTTTATTTTATATCATTTCTAATAATCCTAAACTTTAAT5'
5'TAAGGTAGTAGTATTATAAATTATGGAGGTTTTXAGTGGGAAATAAAATATAGTAAAGATTATTAGGATTTGAAATTATGTAATTGAAAGTAAATGTAGT3'
```

d)

```
                                    TCACCCTTTATTTTATATCATTTCTAATAATCCTAAACTTTAATACATTAACTTTCATTTACATCA5'
5'TAAGGTAGTAGTATTATAAATTATGGAGGTTTTXAGTGGGAAATAAAATATAGTAAAGATTATTAGGATTTGAAATTATGTAATTGAAAGTAAATGTAGT3'
```

seen in Fig. 4, *A* and *B*, pol λ(244–575) had also a clearly diminished intrinsic capacity to bypass the AP lesion compared with the WT (compare *lanes 4* with *5* and *lanes 6* with *7*). Note that neither WT nor mutant pol λ could replicate past the AP site on single-stranded template-primer or on a template bearing a gap as long as 13 nucleotides (see *lanes 2, 3, 8,* and *9* of Fig. 4*A* and quantified in *B*), in agreement with the results previously shown.

These experiments show the following: 1) a pol λ mutant lacking its BRCT and proline-rich domains has an impaired capacity to perform TLS of an AP site in the presence of pol ϵ, and 2) this defect can be attributed to an intrinsic diminished capacity to bypass the lesion, therefore suggesting a role of these domains in facilitating TLS of an AP site by pol λ.

*DNA Polymerase β and DNA Polymerase λ Show Different DNA Gap Size Preference for Translesion Synthesis Past an AP Site in the Presence of DNA Polymerase ϵ*—Next we tested whether DNA gap sizes could influence, in the presence of pol ϵ, TLS of an AP site by pol β, another X family repair polymerase that displays high affinity for very short DNA gaps. A direct comparison between the TLS capacity of β and λ as a function of DNA gap size is presented in Fig. 5.

First, it should be noted that on a single-stranded template-primer and at the same protein concentration (0.25 pmol) pol β could synthesize up to the AP site but pol λ could not (compare *lanes 5* and *6* with *lanes 3* and *4* of Fig. 5*A*). However, the elongation was blocked at the base preceding the lesion. Therefore, it appears that pol β cannot bypass the AP site when acting alone on a single-stranded template-primer. Furthermore, Fig. 5*A* also shows that neither pol λ nor β can perform TLS by utilizing primers created by pol ϵ in a single-stranded context (*lanes 7, 8, 13,* and *14* and quantified in Fig. 5*B*). In the presence of pol ϵ and in agreement with the data shown in Fig. 1, pol λ could easily replicate past the AP site when the gaps were 2 or 4 nucleotides long (*lanes 9–12*).

Interestingly, pol β also showed the capacity to bypass an AP site in the presence of pol ϵ, but only when the gap was 2 nucleotides long; enlarging the gap to 4 nucleotides abolished the TLS capacity of pol β (Fig. 5*A, lanes 15–18*). pol β appeared to be less efficient than pol λ in TLS of a 2-nucleotides gap (see quantifications in Fig. 5, *B* and *C*), which could be due to the superior intrinsic capacity of pol λ to replicate an AP site (12).

The different gap size dependence of TLS between pols λ and β (see also Figs. 7 and 8) corresponds to the preferences of the respective polymerases for undamaged substrates. pol β fills preferentially the gaps of one nucleotide, and its incorporation efficiency decreases with the increase in gap size from 1 to 4 nucleotides (22). To directly investigate the affinity of pol β to

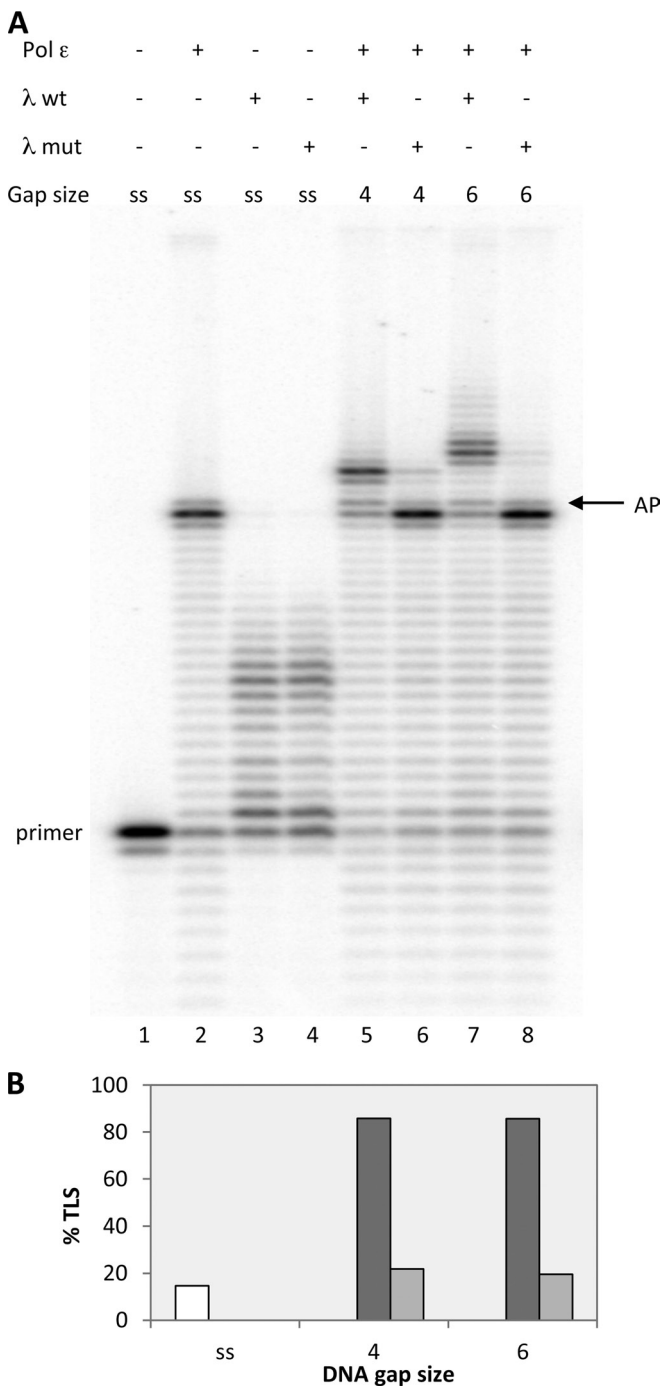


FIGURE 3. **Full-length DNA pol λ is required for the AP site translesion synthesis in the presence of DNA pol ϵ.** Experiments were performed with templates shown in Table 1a. The enzymes and the DNA substrates used are indicated at the *top*. *ss* stands for template-primer with no oligonucleotides hybridized downstream from the AP site, whereas *4* and *6* indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A, lane 1,* no polymerase present. *Lane 2,* reaction incubated for 35 min with 0.025 pmol of pol ϵ. *Lane 3,* reaction incubated with 0.25 pmol of pol λ for 5 min. *Lane 4,* reaction incubated with 0.25 pmol of pol λ(244–575) for 5 min. *Lane 5,* reaction incubated with 0.025 pmol of pol ϵ for 30 min and then 0.25 pmol of pol λ was added and incubation continued for 5 min. *Lane 6,* reaction incubated with 0.025 pmol of pol ϵ for 30 min, and 0.25 pmol of pol λ(244–575) was then added and incubation continued for 5 min. *Lanes 7* and *8,* were as for *lanes 5* and *6,* respectively. The positions of the primer and of the AP site are indicated. *B,* quantification of the data from *A*, expressed as percentage of TLS calculated as described under "Experimental Procedures." *White bar,* pol ϵ alone. *Dark gray bar,* pol ϵ plus pol λ. *Light gray bar,* pol ϵ plus pol λ(244–575).

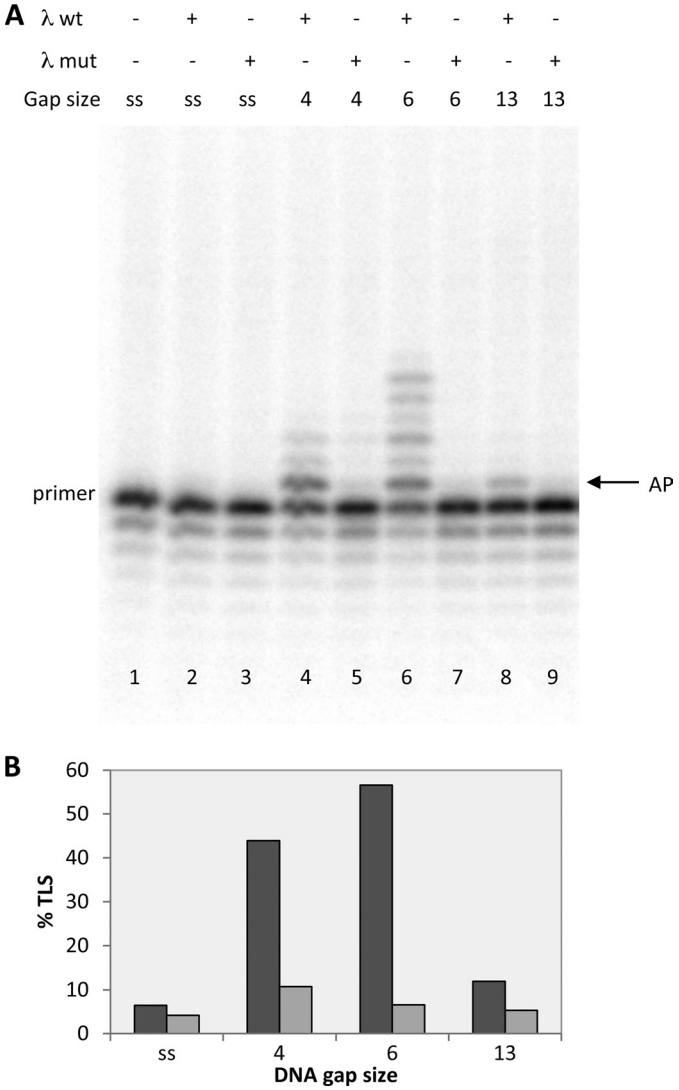


FIGURE 4. **Capacity of the λ(244–575)-truncated form of DNA pol λ to extend from the nucleotide preceding the AP site is impaired.** Experiments were performed with templates shown in Table 1c. The enzymes and the DNA substrates used are indicated at the *top*. ss stands for template-primer with no oligonucleotides hybridized downstream from the AP site, whereas *4, 6,* and *13* indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A, lane 1,* no polymerase present. *Lane 2,* reaction incubated for 5 min with 0.25 pmol of pol λ. *Lane 3,* reaction incubated for 5 min with 0.25 pmol of pol λ(244–575). *Lane 4,* reaction incubated for 5 min with 0.25 pmol of pol λ. *Lane 5,* reaction incubated for 5 min with 0.25 pmol of pol λ(244–575). *Lane 6,* reaction incubated for 5 min with 0.25 pmol of pol λ. *Lane 7,* reaction incubated for 5 min with 0.25 pmol of pol λ(244–575). *Lane 8,* reaction incubated for 5 min with 0.25 pmol of pol λ. *Lane 9,* reaction incubated for 5 min with 0.25 pmol of pol λ(244–575). The positions of the primer and of the AP site are indicated. *B,* quantification of the data from *A*, expressed as percentage of TLS calculated as described under "Experimental Procedures." *Dark gray bar,* pol λ. *Light gray bar,* pol λ(244–575).

the templates-primers used, we measured its binding capacity to substrates containing AP sites in gaps of 1–4, 6, and 8 nucleotides. These substrates were created by first annealing a 66-mer to the 100-mer primer containing the AP site, so that the primer ended at the base preceding the AP site, as depicted in Table 1d. Then the appropriate oligonucleotides were annealed downstream from the lesion to create the substrates mentioned above. The experiment in supplemental Fig. 2

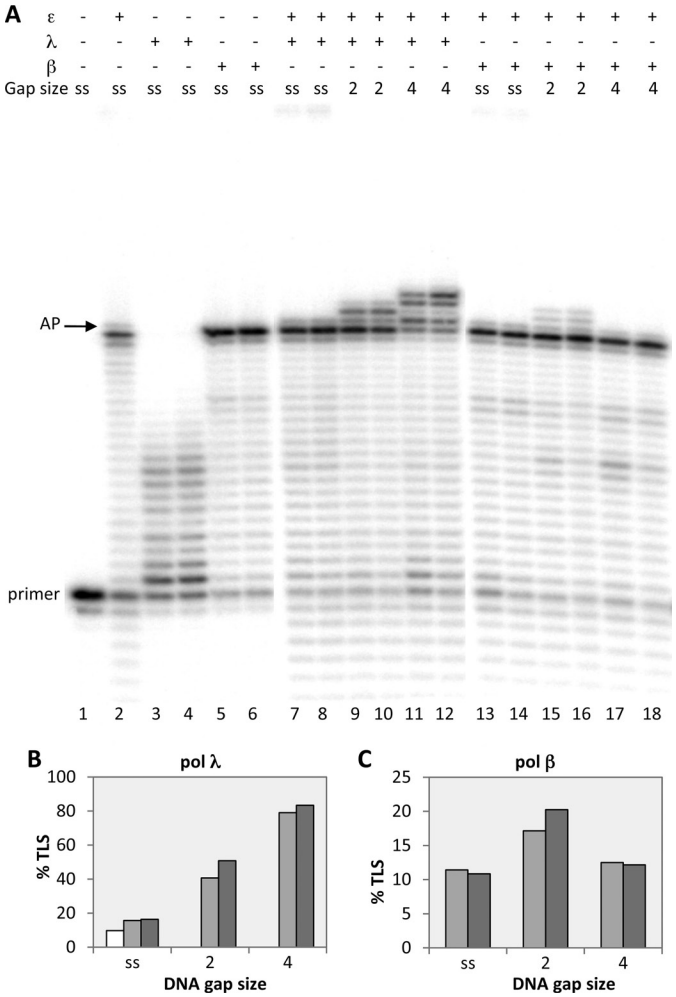


FIGURE 5. **DNA pol β favors smaller gap size than DNA pol λ in TLS of an AP site in the presence of DNA pol ϵ.** Experiments were performed with templates shown in Table 1a. The polymerases and the DNA substrates used are indicated at the *top*. *ss* stands for template-primer with no oligonucleotides hybridized downstream from the AP site, whereas 2 and 4 indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A, lane 1,* no polymerase present. *Lane 2,* reaction incubated for 35 min with 0.025 pmol of pol ϵ. *Lanes 3* and *4,* reactions incubated with 0.25 pmol of pol λ for 5 and 10 min, respectively. *Lanes 5* and *6,* reactions incubated with 0.25 pmol of pol β for 5 and 10 min, respectively. *Lanes 7* and *8,* reactions were incubated with 0.025 pmol of pol ϵ for 30 min, then 0.25 pmol of pol λ were added and the incubation continued for 5 and 10 min, respectively. *Lanes 9–12*: as for *lanes 7* and *8*. *Lanes 13* and *14,* reactions were incubated with 0.025 pmol of pol ϵ for 30 min, then 0.25 pmol of pol β were added and the incubation continued for 5 and 10 min, respectively. *Lanes 15–18*: as for *lanes 13* and *14*. The positions of the primer and of the AP site are indicated. *B,* quantification of the data from *A* with pol λ, expressed as percentage of TLS calculated as described under "Experimental Procedures." *White bar,* pol ϵ alone. *Light gray bars,* incubation with pol ϵ and then pol λ added for 5 min. *Dark gray bars,* incubation with pol ϵ and then pol λ added for 10 min. *C,* quantification of the data from *A* with pol β, expressed as percentage of TLS and calculated as described under "Experimental Procedures." *Light gray bars,* incubation with pol ϵ and pol β added for 5 min. *Dark gray bars,* incubation with pol ϵ and pol β added for 10 min.

shows that the amount of pol β bound to the substrate was highest with a 1-nucleotide gap and diminished with increasing gap size. The reduction in binding efficiency parallels the decrease of TLS by pol β in the presence of pol ϵ, indicating that the TLS capacity of the enzyme is directly correlated with its binding capacity at gaps.

*Nucleotide Insertion Opposite the AP Site by Polymerases β and λ during Gap-directed Translesion Synthesis*—To determine the identity of the nucleotide inserted opposite the synthetic abasic site by pol β and λ, we have ligated the elongated primers to the downstream oligonucleotide and amplified the full-length products by PCR (see "Experimental Procedures"). Because Pfu DNA polymerase is blocked by the synthetic AP site (data not shown), only the newly synthesized DNA strand is amplified during the PCR. As shown in supplemental Table 1, sequencing of individual clones revealed that pol β TLS of the AP site in a 2-nucleotide DNA gap context resulted in incorporation of dAMP opposite the AP site in all the products sequenced, according to the proposed model of bypass of an abasic lesion by pol β (26). However, a 4-nucleotide DNA gap filling reactions by pol λ resulted in single or double deletions opposite the lesion, according to the known misalignment capacity of the enzyme (27). It should be noted that, in our template sequence, the AP site is followed by a run of 4 thymine residues; therefore, incorporation of dAMP by pol λ opposite the lesion followed by template-primer slippage and annealing to a downstream thymine can lead to the observed pattern.

*Translesion Synthesis of an AP Site by DNA Polymerase η, Acting Alone or in the Presence of DNA Polymerase ϵ, Is Not Influenced by the Presence of DNA Gaps*—Next, we investigated the capacity of the human pol η to perform TLS of an AP site either in the presence of pol ϵ or when acting alone (Fig. 6). 0.25 pmol of pol η, incubated with single-stranded template-primer in a running start reaction, synthesized up to the AP site and, differently from pol ϵ, efficiently incorporated opposite the lesion (compare *lane 4* with *lane 2* of Fig. 6*A* and data not shown). When the reaction was performed in the presence of pol ϵ, pol η performed TLS with the single-stranded template primer, as indicated by the increase in incorporation in front of the lesion and the appearance of some full-length products (see *lane 9* of Fig. 6*A*). However, this TLS was diminished in the presence of gaps of 2, 4, and 6 nucleotides, and extension past the lesion was not affected by the increase in their size (see *lanes 10* to *12* of Fig. 6*A*). This result was different from what was observed with pol λ, where no TLS was observed in the presence of pol ϵ with a single-stranded template-primer (see *lane 5* of Fig. 6*A*) whereas efficient gap filling was performed with DNA gap sizes of 2, 4, and 6 nucleotides (see *lanes 6* to *8* of Fig. 6*A*).

We have also studied the TLS capacity of pol η when acting alone on gaps created in a template-primer where the primer is a 45-mer with a 3′OH at the base preceding the lesion. As shown in Fig. 6*B*, pol η can perform TLS on the single-stranded template (*lane 7*) but to a lesser extent with gaps of 1, 2, 4, and 6 nucleotides and with no increase in efficiency with the increasing gap size (see *lanes 8–11*). Conversely, TLS by pol λ was stimulated with gap size increasing from 2 to 6 nucleotides (Fig. 6*B*, *lanes 4–6*). Note that pol η showed little TLS also with gap size of 1 and 2 nucleotides that are optimal for TLS by pol β (see Fig. 8). Taken together, these results show that, differently from pol λ, TLS of an AP site by pol η is not stimulated by the presence of DNA gaps.

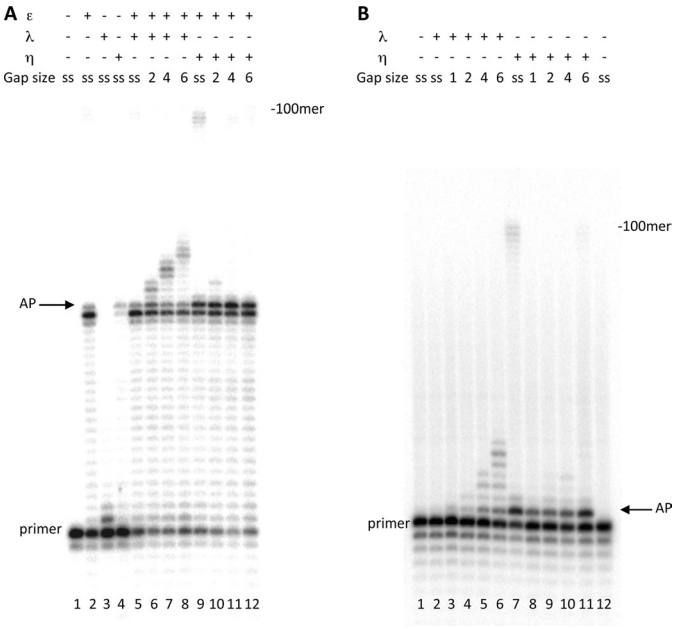


FIGURE 6. **Influence of DNA gap sizes on translesion synthesis of an AP site by DNA pol λ and η.** Experiments were performed with templates shown in Table 1. The enzymes and the DNA substrates used are indicated at the *top*. *ss* (single-stranded) stands for template-primer with no oligonucleotides hybridized downstream from the AP site, whereas *1, 2, 4,* and *6* indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A,* experiments with templates in Table 1a. *Lane 1,* no polymerase present. *Lane 2,* reaction incubated for 35 min with 0.025 pmol of pol ϵ. *Lane 3,* reaction incubated for 5 min with 0.25 pmol of pol λ. *Lane 4,* reaction incubated for 5 min with 0.25 pmol of pol η. *Lanes 5–8,* reaction was incubated with 0.025 pmol of pol ϵ, and then 0.25 pmol of pol λ were added, and the reaction was continued for 5 min. *Lanes 9–12,* reaction was incubated with 0.025 pmol of pol ϵ, and 0.25 pmol of pol η were then added, and the reaction was continued for 5 min. *B,* experiments with templates in Table 1c primed with the 45-mer terminating one nucleotide before the Ap site. *Lanes 1* and *12,* no polymerase present. *Lanes 2–6,* reactions incubated 5 min with pol λ. *Lanes 7–11,* reactions incubated 5 min with pol η. The positions of the primers, of the AP site, and of the 100-mer full-length product are indicated.

*PCNA and RPA Do Not Influence the Gap Size Preference of DNA Polymerases λ and β to Perform Translesion Synthesis of an AP Site in the Presence of DNA Polymerase ϵ*—The processivity factor PCNA and the single-stranded DNA-binding protein RPA play a fundamental role in DNA replication, repair, and recombination (2, 5). Therefore, we studied whether the TLS catalyzed by pols λ and β at DNA gaps in the presence of pol ϵ could be influenced by human PCNA and RPA. To this aim we performed experiments by adding 1.2 pmol of PCNA and 0.25 pmol of RPA. With respect to the 0.15 pmol of template-primer used in the study, the RPA concentration corresponds to roughly one molecule of RPA for 30 nucleotides of single-stranded DNA.

Fig. 7 shows the results with pol λ. As can be seen with a wide range of DNA gaps (Fig. 7*A*), pol λ performed TLS with an efficiency dictated by the size of the DNA gaps. This efficiency increased with gaps from 1 to 4 nucleotides, remained constant with 6 and 8 nucleotides, and then declined to an almost undetectable level with the gap of 13 nucleotides, as quantified in Fig. 7*B*. As shown previously, also in this reaction containing both PCNA and RPA, the TLS products length essentially matched the size of the gaps, although low levels of strand displacement could also be detected.

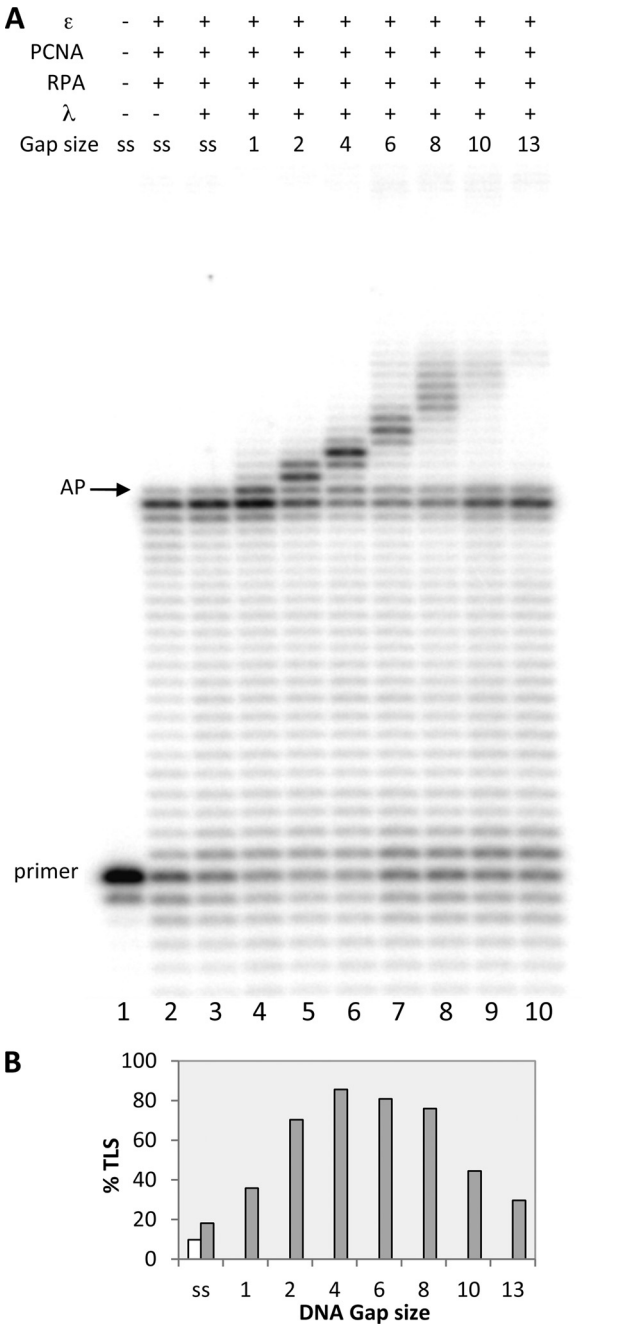


FIGURE 7. **PCNA and RPA have no effect on gap size specificity of TLS of an AP site by DNA pol λ in presence of DNA pol ϵ.** Experiments were performed with templates shown in Table 1a. The proteins and the DNA substrates used are indicated at the *top*. *ss* stands for template-primer with no oligonucleotides hybridized downstream from the AP site, whereas *1, 2,* and *4, 6, 8, 10,* and *13* indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A, lane 1,* no polymerase present. *Lane 2,* reaction incubated for 35 min with 0.025 pmol of pol ϵ, 1.2 pmol of PCNA, and 0.25 pmol of RPA. *Lanes 3–10,* the reactions were incubated for 30 min with 0.025 pmol of pol ϵ, 1.2 pmol of PCNA, and 0.25 pmol of RPA, and then 0.25 pmol of pol λ were added, and the incubation was continued for 5 min. The positions of the primer and of the AP site are indicated. *B,* quantification of data from Fig. 6*A*, expressed as percentage of TLS calculated as described under "Experimental Procedures." *White bar,* reaction without pol λ. *Gray bar,* complete reaction.

Fig. 8 shows the results with pol β. Ast can be seen, the TLS by pol β is restricted to gaps of 1 and 2 nucleotides, becoming almost undetectable with a gap of 4 nucleotides or longer (Fig.

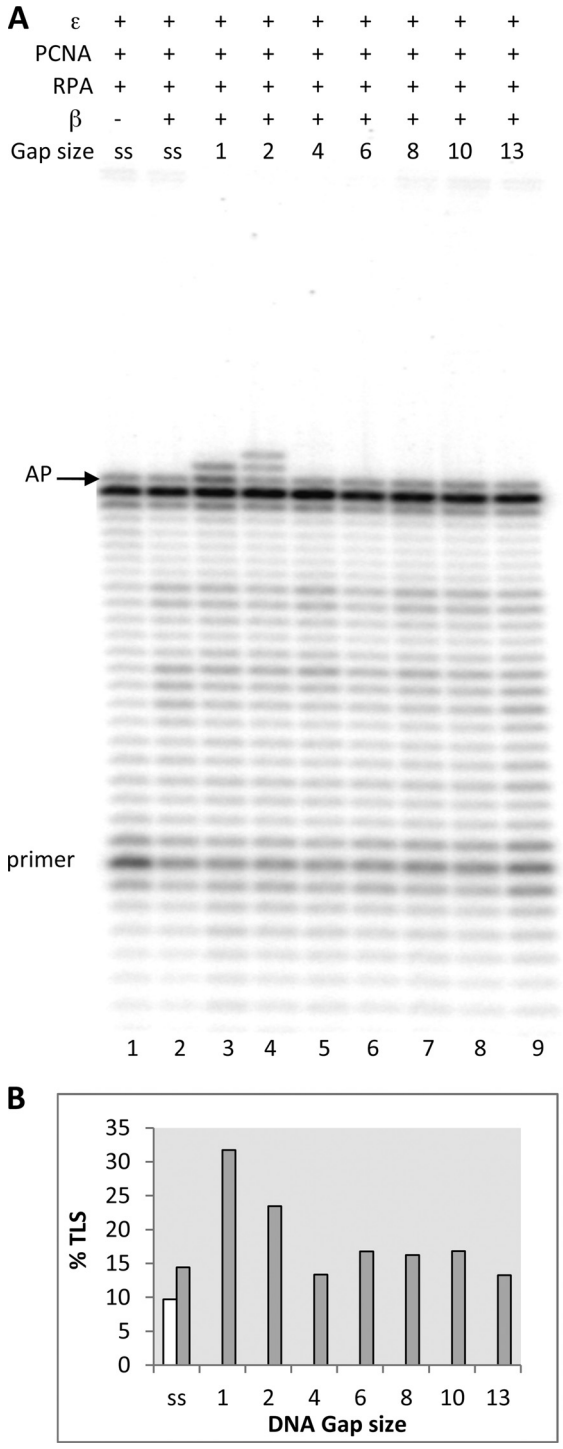


FIGURE 8. **PCNA and RPA have no effect on gap size specificity of TLS of an AP site by DNA pol β in the presence of DNA pol ϵ.** Experiments were performed with templates shown in Table 1a. The proteins and the DNA substrates used are indicated at the *top*: *ss* stands for template-primer with no oligonucleotides hybridized downstream from the AP site, whereas *1, 2,* and *4, 6, 8, 10,* and *13* indicate the length of gap regions between the AP site and the oligonucleotide hybridized downstream. Assays were carried out as described under "Experimental Procedures." *A, lane 1,* reaction incubated for 35 min with 0.025 pmol of pol ϵ, 1.2 pmol of PCNA, and 0.25 pmol of RPA. *Lanes 2–9*: the reactions were incubated for 30 min with 0.025 pmol of pol ϵ, 1.2 pmol of PCNA, and 0.25 pmol of RPA, and then 0.25 pmol of pol β were added, and the incubation was continued for 5 min. The positions of the primer and of the AP site are indicated. *B,* quantification of data from Fig. 7*A*, expressed as percentage of TLS calculated as described under "Experimental Procedures." *White bar,* reaction without pol β. *Gray bar,* complete reaction.

8*A*). In agreement with the experiments described previously, the efficiency of TLS by pol β was lower when compared with that observed with pol λ (quantified in Fig. 8*B*), and no strand displacement was observed.

## DISCUSSION

pols δ and ϵ are the two eukaryotic polymerases that replicate DNA. Evidence in yeast supports the conclusion that pol δ is primarily responsible for copying the lagging strand, whereas pol ϵ primarily copies the leading strand (3). In addition, these polymerases also participate in DNA repair processes such as nucleotide excision repair (NER), long patch base excision repair (LP-BER), and mismatch repair (2).

Apurinic or apyrimidinic sites are the most frequent spontaneous lesions in DNA. A number of polymerases, belonging mainly but not exclusively to the Y family polymerases, can perform *in vitro* TLS of an AP site (9, 28). pols λ and β, two polymerase of the X family believed to be implicated in re-synthesis steps of DNA repair, can also bypass an abasic site (12).

Although much information is available in the literature concerning the capacity of pol δ to deal with an AP site (11, 29), such information is scarce for pol ϵ, particularly for the human enzyme. In a recent publication, we have shown that *in vitro* elongation by human pol ϵ stopped predominantly at the base preceding the lesion with roughly 10% of residual incorporation opposite to it (14).

As indicated in the Introduction, it is generally accepted that during TLS specialized polymerases replace arrested replicative polymerases at lesions to allow bypass. We have investigated the ability of human pols λ, β, and η to perform TLS of an AP site in the presence of short DNA gaps created by the arrest of human pol ϵ at the lesion. To this aim we have set up a system in which pol ϵ synthesizes on a DNA oligonucleotide template-primer leaving gaps with lengths spanning from 1 to 13 nucleotides because of stalling at the AP site (Table 1).

pol λ cannot bypass an AP site in the presence of pol ϵ if a single-stranded stretch of 35 nucleotides is present downstream from the lesion, but it can do it with gaps from 1 to 10 nucleotides, with a maximum efficiency around 4 to 6 and with almost no TLS with a gap of 13 nucleotides (Figs. 1, 3, 5, and 7). Furthermore, our data indicate the following: (*a*) pol λ can bypass an AP site by using the 3′OHs generated by the arrest of pol ϵ at the lesion (supplemental Fig. 1); (*b*) it can act alone to bypass the AP site only when the lesion is present in a gap context and, importantly, with a gap preference similar to the one observed in the presence of pol ϵ (Fig. 2); (*c*) the BRCT and proline-rich domains of the pol λ are required for efficient TLS of the lesion (Figs. 3 and 4). These results show that pol λ can replace pol ϵ and bypass an AP site utilizing 3′OHs created by the arrest of pol ϵ at the base preceding the lesion or opposite to it. Most interestingly, this switch can take place only on short DNA gaps, whose size strongly influences the efficacy of the process, and bypass of an AP site requires the presence of the BRCT and proline-rich domain of the pol λ. We attempted to further characterize this scenario by investigating directly the binding capacity of pol λ with the gapped DNA substrates used in this study. In contrast to experiments with pol β (see below), we were unable to detect stable interaction between pol λ and

DNA substrates under a variety of conditions. However, our results are in full agreement with the gap size preference of pol λ on undamaged DNA recently published (22).

pol β can also catalyze TLS of an AP site in the presence of pol ϵ, but only if the gaps have a size of 1–3 nucleotides, with an efficacy of 1 > 2 > 3 nucleotides and with no bypass of an AP site at gaps longer than 3 nucleotides (Figs. 5 and 8 and data not shown). This preference parallels the binding affinity of pol β for the DNA substrates, as can be seen in supplemental Fig. 2, and it is in agreement with the fact that its incorporation efficiency for undamaged substrates is the highest with 1 nucleotide gap and then decreases with the increase of gap size (22). The efficiency of TLS by pol β appears to be reduced compared with the one displayed by pol λ, likely because of the superior intrinsic capacity of pol λ to replicate an abasic site (12).

Sequencing of the replication products revealed that pol β exclusively inserts dAMP in front of the abasic site in the context of a 2-gap substrate, whereas pol λ bypass of an AP site in a 4-gap substrate induces single or double deletions (supplemental Table 1). Insertion of dAMP by pol β is in accordance with a previous model of pol β translesion synthesis of an abasic site that revealed its predisposition to inserting a nucleotide complementary to the first downstream templating base, which is a thymine in our template sequence (26). The observed behavior of pol λ fits with the scrunching gap-filling model derived from crystal structures of the ternary DNA-pol λ-dNTP complex (30).

pol η was also proficient in TLS of an AP site when acting alone or in the presence of pol ϵ. However its TLS capacity was not stimulated by the presence of DNA gaps (Fig. 6).

PCNA increases the processivity of the replicative pol δ (2), but its capacity to stimulate or not the processivity of pol ϵ remains controversial, possibly depending on the type of DNA substrates and experimental conditions used (31–34). Furthermore, PCNA has been shown to stimulate TLS of an AP site by pol λ when placed on a 73-mer template, 31 nucleotides away from the 17-mer primer (35).

Because PCNA and RPA play a fundamental role in DNA transactions such as DNA replication, repair, and recombination, we set up to study whether the TLS catalyzed by pols λ and β at DNA gaps in the presence of pol ϵ would be influenced by these proteins. When compared with previous figures, Figs. 7 and 8 show that PCNA and RPA had no effect. In addition, Figs. 7 and 8 summarize the major findings of this work by showing that human pols λ and β can perform TLS of an AP site in the presence of human pol ϵ, PCNA, and RPA only in DNA gaps no longer than 10 nucleotides. However, the two polymerases show distinctly different DNA gap size preference and efficiency in performing such TLS.

Accordingly, our results suggest the simple model shown in Fig. 9, in which the different role of pol β and λ in extending primers generated by pol ϵ arrested at an AP site can be visualized. When long single-stranded DNA stretch is present downstream from the lesion, neither pol has sufficient affinity for the DNA substrate to displace pol ϵ and continue DNA synthesis past the AP site (Fig. 9*a*). When the gap downstream from the lesion is only 1 or 2 nucleotides long, both pols β and λ could use their affinity for such gaps to bind and perform TLS (Fig. 9*b*). If the gap size is between 4 and 10 nucleotides, only pol λ would have the capacity to bind and extend past the lesion (Fig. 9*c*). If the gap size is larger than 13 nucleotides, neither λ nor β could bind productively, and we are back to the initial situation with single-stranded DNA downstream from the AP site (Fig. 9*d*).

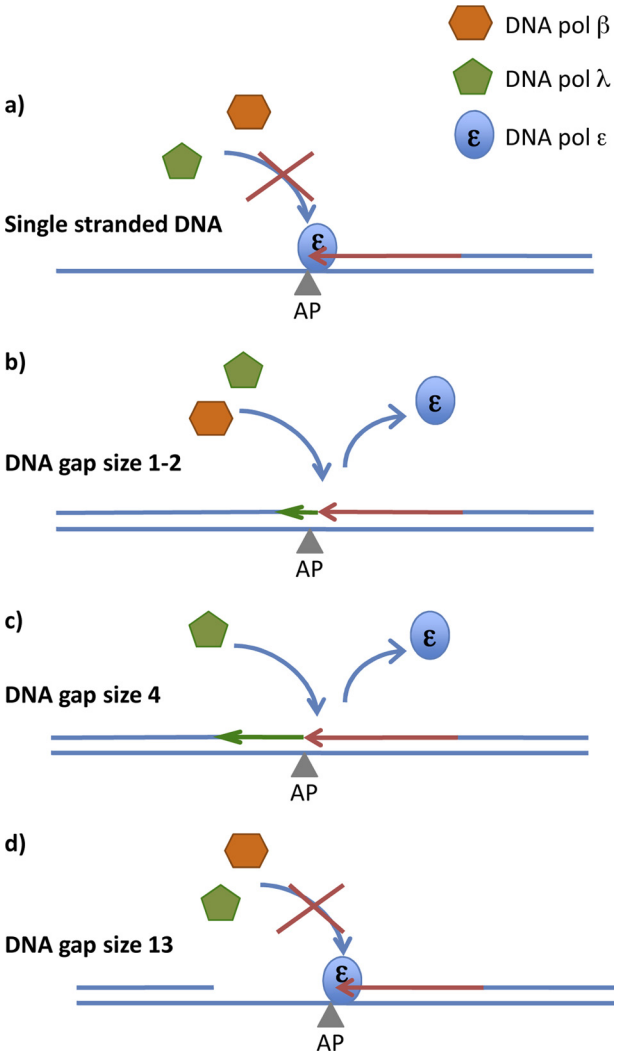


FIGURE 9. **Tentative model of TLS over an AP site by DNA pol λ and β in the presence of DNA pol ϵ.** For details see text.

What might be the physiological significance of our *in vitro* observations? At least two possibilities exist. The first concerns DNA repair, namely NER and LP-BER pathways. In mammalian cells, it has been shown that pol ϵ can fill the gap of about 27 nucleotides that is produced during NER in a reaction that includes PCNA and RPA (36). The redundant roles of pols δ and ϵ have been confirmed during NER of a defined lesion reconstituted with recombinant or highly purified factors *in vitro* (37). The requirement of pol ϵ and RPA in the re-synthesis step of NER in human cells has been further demonstrated recently, together with another pathway involving pols δ and κ (38). Because AP sites are among the most frequent endogenous lesions, it is possible that the removal of the damaged 27-mer during NER uncovers an AP site in the DNA template sequence of the gap to be filled. This gap filling will be per-

formed first by pol ϵ up to the lesion and subsequently by pol λ or β depending on the distance of the AP site from the 5′ end of the gap.

A similar situation can arise during the long patch BER pathway. It has been suggested that either pol ϵ or pol δ catalyzed elongation during long patch BER synthesis (39), and it has been shown that both polymerases can participate in the resynthesis step of long patch BER (40). Furthermore, it is now known that two clustered DNA lesions enhance the mutagenicity of individual lesions (for review see Ref. 41). This observation suggests that the delayed repair at one lesion because of the initiation of repair on the opposite strand can lead to mutagenic TLS of the unrepaired damage. In mammalian cells, pols ϵ, β, or λ could sequentially perform the replication of an AP site during LP-BER of clustered DNA lesions in the way suggested by our model.

The second possibility concerns gap-directed TLS in connection with DNA replication where small single-stranded DNA gaps accumulate along replicated duplexes. Such gaps may arise either by re-priming of the leading strand (42) or the initiation of a new Okazaki fragment (42, 43). Gap-directed lesion bypass has the advantage that the TLS may be separated from the fork progression, and in fact, recent results suggest that a considerable fraction of TLS occurs in the $G_2$/M phase of the cell cycle, when DNA replication has essentially completed (44). As in repair-dependent, gap-directed TLS, the nature of the lesion and the size of the gap may determine which polymerase could be best suited for the bypass.

In summary, although based on *in vitro* experiments, our study suggests the existence of DNA gap-directed TLS of an abasic site involving human pols ϵ, λ, and β, and it might serve as a working model for further investigations.

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# Unpublished results

## Translesion synthesis fidelity assay

In the following, the unpublished procedures and results of a side-project initiated in an attempt to clarify the true relevance of Pol λ in repair of 8-oxo-G damage *in vivo* are presented.

## Introduction and background of the approach

To approach the question whether Pol λ is truly relevant in repair of 8-oxo-G *in vivo*, a procedure that has been established by Zvi Livneh's lab in Israel was used, as outlined in the following (107). In short, a gapped plasmid with a single-stranded region containing an 8-oxo-G lesion was constructed. This gapped plasmid was transfected into mammalian cells that were harvested after an appropriate incubation time. The single-strand gap opposite the lesion was closed by the cellular TLS and repair machinery of the cells. After an alkaline lysis step to eliminate any not fully double-strand plasmid (i.e. all gapped plasmids in which the gap has not been completely closed by the cellular machinery), the plasmid was extracted and purified. To determine how accurately the gap opposite the lesion had been closed, different approaches were pursued from this point forward. In possibility number one, the original assay devised by Zvi Livneh, the extracted plasmid mix was transformed into *E.coli mutY*$^{-/-}$ and the plasmids in the resulting colonies were subject to sequencing analysis, revealing what nucleotide was inserted opposite the lesion in the mammalian cells in the first place. For this, it is important to keep in mind that the *E.coli* strain used for the transformation is *mutY*$^{-/-}$ as the bacterial MutY would otherwise have removed any A incorporated opposite 8-oxo-G and thus have led to an incorrectly low rate of transversion mutations. As a second possibility to determine the rate of correct 8-oxo-G bypass, the 8-oxo-G lesion was introduced into a sequence context that allowed cleavage by a restriction enzyme in the case of reconstitution of the correct CG base pairing, whereas this restriction site was lost upon the occurrence of a CG → AT transversion mutation. Any colonies that had lost this restriction site were sent for sequencing to determine the nature of the mutation. The third approach was to use an allele-specific PCR to assay for the rate of correct CG versus incorrect AT base pair (140). This assay is based on the fact that a SNP can be detected in a real-time PCR setup. The allele specificity of the PCR reaction was conferred by the use of two practically identical primers that only differ in the 3' end, which matched either the correct or the incorrect nucleotides inserted opposite the 8-oxo-G lesion. The idea behind it was the assumption that, when two reactions only differing in the SNP-detection-primer were carried out in parallel, only perfectly matching primer ends were extended and thus only the matching SNP carrying plasmid was amplified. This could be monitored in a real-time cycler.

In order to address the nature of the Pol important for the non-mutagenic 8-oxo-G translesion synthesis, a knockdown of different Pols by siRNA was performed. They included 14 Pols from four mammalian Pol families, Pols γ, θ and n of the family A, the Pols α, δ, ε and ζ from the family B, the Pols β, λ and μ from the family X and the Pols κ, η, ι and Rev1 from the Y family of TLS Pols (see also *Table 1* for details of the Pols).

## Materials and methods

### Production of the substrates for the assay

In order to establish the translesion synthesis fidelity assay developed by Zvi Livneh's laboratory, the plasmid pSKSL and the *E.coli muty-/-* strain were received from the Livneh lab. Next chemically very competent *E.coli muty-/-* were produced. The oligonucleotides for the production of gapped plasmids carrying either a normal G or an 8-oxo-G in the gap were designed in a way that the restriction enzyme FauI, which cuts the pSKSL backbone in one single site, would cut only if the correct CG base pair was established at the 8-oxo-G carrying site and not if a AT transversion had occurred (*Figure 4)*.

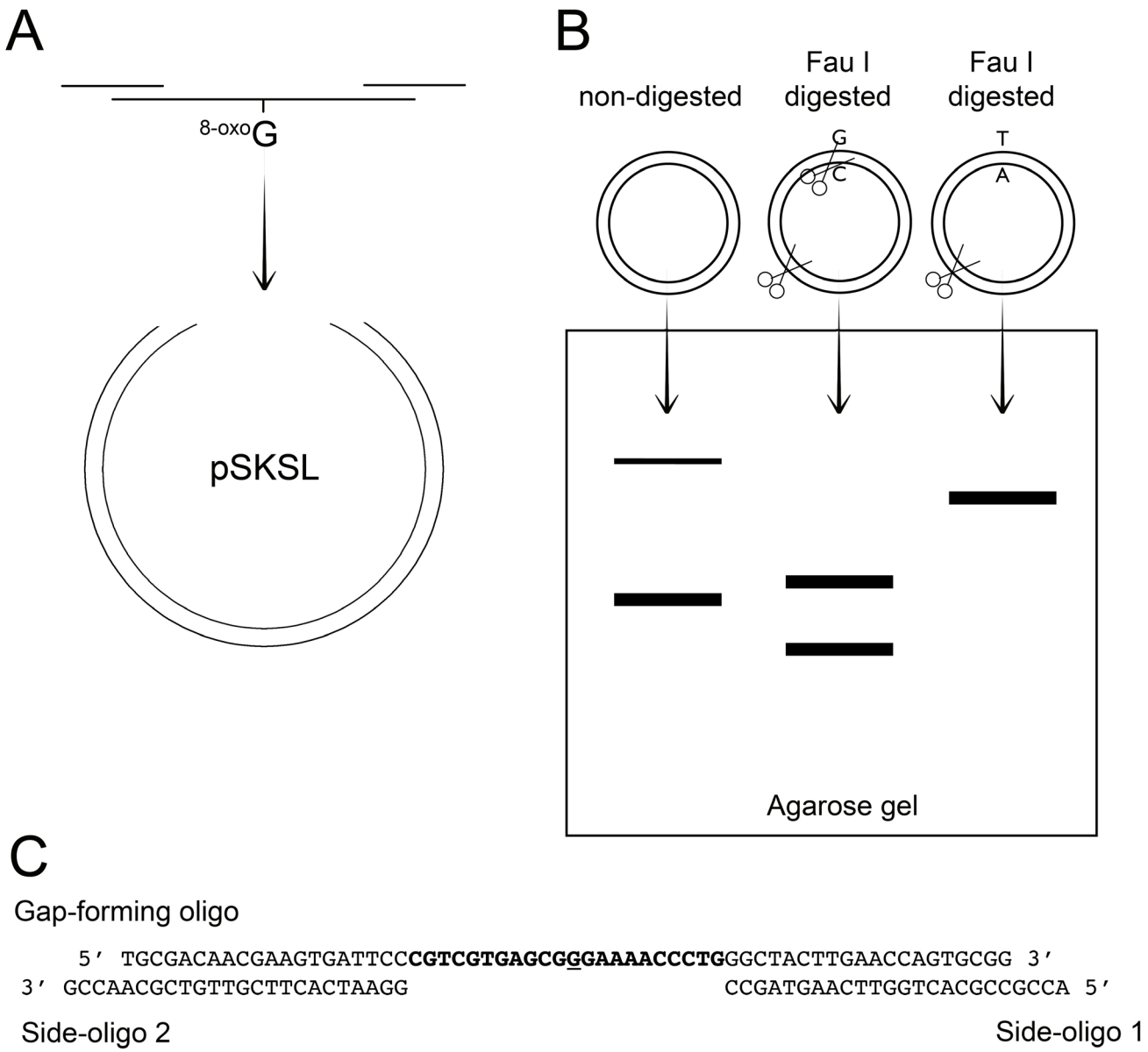


***Figure 4: Schematic presentation of the gapped plasmids.***
A) The plasmid pSKSL is linearized and an insert forming a gap with an 8-oxo-G in the single-stranded region is ligated into it, thus yielding a circular gapped plasmid. B) This panel demonstrates the products of the FauI restriction digestion of plasmid DNA obtained by mini-prepping bacterial colonies. When a correct CG base pairing is present at the site of the original 8-oxo-G lesion, FauI cuts once in the vector backbone and once at the site of the lesion, giving rise to 2 linear plasmid fragments. If the 8-oxo-G site has suffered any kind of mutation, e.g. a AT transversion as shown here, the FauI restriction site at the lesion is lost, thus yielding only linearized plasmid molecules. C) The sequence of the insert formed by 3 oligonucleotides is displayed. The underlined G represents the site of the 8-oxo-G in the damage-carrying oligonucleotide. The FauI restriction site is only present when a correct CG base pairing is present.

In total, the following constructs were produced:

- Gapped plasmid with normal G in the single-strand region
- Gapped plasmid with an 8-oxo-G in the single-strand region
- Fully double-strand, circular covalently closed plasmids with an 8-oxo-G:A mismatch (mimicks the plasmid after incorrect gap-filling has taken place)
- Fully double-strand, circular covalently closed plasmids with CG and AT inserts (represents the final product from correct and wrong insertion, followed by excision of 8-oxo-G, leading to the generation of correct CG or incorrect AT base pairing)

## Set-up of the allele-specific PCR

In order to determine, whether the allele-specific PCR (AS-PCR) was a feasible option to address the identity of nucleotides inserted opposite the 8-oxo-G, reactions using 'model plasmids' were performed. The 'model plasmids' were the pSKSL carrying fully double-stranded CG or AT containing inserts that reflect the final products of successful gap-filling reaction and 8-oxo-G repair. The AS-PCR proved to be feasible on those plasmids, as the assay could clearly discriminate between AT or CG carrying pSKSL. Furthermore, the assay could also discriminate clearly between pSKSL carrying an 8-oxo-G:C from an 8-oxo-G:A base pairing, which represent the reaction intermediates after gap-filling but still before the 8-oxo-G base has been excised. Furthermore, the assay also worked in the extracts from HeLa cells transfected with the gapped plasmids. Thus, the proof-of-principle for using the AS-PCR for the determination of the nucleotide inserted opposite the 8-oxo-G could be given.

## Assessment of the FauI restriction approach

The original assay by Livneh uses transformation of the plasmid extracts from mammalian cells into *muty-/-* bacteria, mini-prepping of the growing colonies and subsequent sequencing to determine the nucleotide inserted opposite the lesion in the gap. In order to be able to screen the bacterial mini-preps before sequencing for the ones that do not have the correct CG containing base pairing at the site of the lesion, the oligonucleotide was designed in a way that mutation of the correct CG base pair would lead to the loss of a FauI restriction site (see *Figure 4)*.

# Results

## Transformation of gapped plasmids into mammalian cells

For mammalian cell transfection with the gapped plasmids, initially two different incubation times, 7 and 24 hours, were chosen. This was due to following reasoning: firstly, Avkin *et al.* used the 7h time-point in their publication because MutYH is not supposed to excise wrongly incorporated A opposite 8-oxo-G at this early time point. This was important for the approach, as the aim was to determine the *primary* translesion synthesis fidelity, so it was a prerequisite for the analysis that any wrongly incorporated A opposite 8-oxo-G remain present until the analysis and be not excised by MutYH, possibly yielding a wrongly lower mutational insertion rate. Secondly, it was assumed that when given enough time – as for instance 24h – the whole repair pathway (gap-closure, excision of eventual wrongly incorporated A by MutYH, subsequent (possibly correct) gap-filling and repair of 8-oxo-G on the opposite strand) could be performed and yield completely repaired plasmids that do not carry any lesion anymore. Restriction digestion of plasmids from *muty-/-* colonies obtained from incubations of the gapped plasmids in HeLa cells for 7 or 24 hours revealed the presence of a mutation in 14.34% and 15% of 75 and 80 preps analyzed (*Table 2*). Sequencing of the mutated preps revealed CG → AT transversions being present in 7.5% and 10.22% out of all the preps, respectively. The mutated plasmids not showing a CG → AT transversion displayed mostly small deletions. These data are slightly lower but still in line with the mutation rate established by Avkin *et al.*, who saw 16% of wrong A incorporation after 7h incubation in H1299 cells when analyzing a total of 60 preps by sequencing. These results demonstrated the reproducibility of the assay in our hands. Furthermore it can be said that the restriction analysis for the screening of preps to be sequenced was clearly proven to be helpful for this approach, as they significantly lowered the number of plasmids in need for sequencing.

The next step was to analyse the extracts for the 24h incubation time-point from *Table 2* with the AS-PCR to see whether the results from the different approaches (restriction, sequencing and AS-PCR) were comparable. The AS-PCR (normalized to a primer-pair annealing to a different region of the pSKSL than the allele-specific ones) showed 6.35% (+/- 1.25%) of AT transversions (*Table 3)*. Moreover, the result from simultaneous transfection of normal G containing gapped plasmid into the HeLa cells yielded almost 100% of correct CG base pairing, which was to be expected. These results demonstrated that the results from the AS-PCR are quite consistent with the ones from the actual sequencing.

***Table 2: Mutational analysis by restriction digestion versus sequencing****

| | 24h incubation of plasmids in HeLa | | 7h incubation of plasmids in HeLa | |
|---|---|---|---|---|
| Extract | Restriction analysis (% of mutated plasmids) | GC→TA transversions (Sequencing) | Restriction analysis (% of mutated plasmids) | GC→TA transversions (Sequencing) |
| 1 | 3/20 = 15% | 0/20 = 0% | 5/26 = 19.2% | 4/26 = 15.4% |
| 2 | 2/20 = 10% | 1/20 = 5% | 4/29 = 13.8% | 3/29 = 10.3% |
| 3 | 3/20 = 15% | 2/20 = 10% | 2/20 = 10% | 1/20 = 5% |
| 4 | 4/20 = 20% | 3/20 = 15% | - | - |
| Mean | 15% | 7.5% TA | 14.34% | 10.22% |

*Extracts from mammalian cells transfected with the 8-oxo-G bearing gapped plasmid for 7h and 24h, respectively, were transformed into *E.coli myh-/-*. Single colonies were picked and plasmid isolation was performed. The plasmid preparations were subject to restriction digestion with FauI and analysed on an 0.8% agarose gel. The percentage of preparations yielding only linear DNA is shown in columns 2 and 4 for the 24h and 7h incubations, respectively. Subsequently the region of the lesion-bearing insert of plasmids yielding only linear DNA after digestion was sequenced. The CG → AT transversions in relation to total amount of preparations analysed are shown in columns 3 and 5, respectively.

***Table 3: Comparison of the AS-PCR to restriction analysis and sequencing****

| Cells | Plasmid | AS-PCR %TA (normalized) | Restriction analysis (% mutated plasmids) | GC→TA transversions (Sequencing) |
|---|---|---|---|---|
| HeLa | 8-oxo-G | 6.35 (+/- 1.25) | 15% | 7.5% TA |
| HeLa | Normal G | 0.04 (+/- 0.00) | - | - |

*The AS-PCR was carried out with extracts from 3 independent transfection experiments, and each of them was analysed 3 times independently. The mean (+/- SD) is shown in comparison with the results from the restriction analysis and the sequencing (from Table 2)

For the next experiments a 7h incubation of gapped plasmids in the mammalian cells was chosen, in order to see the initial incorporation fidelity opposite 8-oxo-G in different Pol knockdown backgrounds, as mentioned above. The results of the initial (single) experiments in HeLa with knockdown of Pols β, θ and η, as detected by AS-PCR, restriction analysis and sequencing are summarised in *Table 4*. A general tendency seems to be that the AS-PCR yields consistently lower numbers of AT transversions as the sequencing results. It remains to be discussed further, which of the two approaches can be trusted more and if those results show any significant differences whatsoever.

As the knockdown of Pol λ was still causing problems at that time, we decided to check the translesion synthesis fidelity in $\lambda^{-/-}$ and b/$\lambda^{-/-}$ MEFs. Additionally, MEFs stably transfected with Pol λ wt and Pol λ T553A (a phospho-defective mutant) were evaluated in the same set-up. Results from the initial (single) experiments evaluated by AS-PCR are summarised in *Table 5*. Those results do not show any striking differences between the cell lines either. In conclusion it seems, that there are no big differences in the initial translesion synthesis step in the different Pol backgrounds.

***Table 4: Results from AS-PCR, restriction analysis and sequencing for knockdown of Pols β, θ and η.***

| Cells | siRNA | Plasmid | AS-PCR %TA (normalized) | Restriction analysis (% mutated plasmids) | GC→TA transversions (Sequencing) |
|---|---|---|---|---|---|
| HeLa | Non-specific | 8-oxo-G | 7.8 | 10/49 = 20.4% | 6/49 = 12.2% |
| | Non-specific | Normal G | 0.1 | - | - |
| HeLa | Polβ | 8-oxo-G | 9.1 | 5/42 = 11.9% | 5/42 = 11.9% |
| | Polβ | Normal G | 0.2 | - | - |
| HeLa | Polθ | 8-oxo-G | 7.9 | 9/48 = 18.75% | 4/47 = 8.5% |
| | Polθ | Normal G | 0.1 | - | - |
| HeLa | Polη | 8-oxo-G | 8.6 | 12/39 = 30.7% | 7/39 = 17.9% |
| | Polη | Normal G | 0.1 | - | - |

***Table 5: Results from transfection of gapped plasmids into different MEF cell lines for 7h, analysed by AS-PCR.***

| Cells | Genotype | Plasmid | %TA (normalized) |
|---|---|---|---|
| MEF | +/+ | 8-oxo-G | 11.0 |
| | +/+ | Normal G | 0.1 |
| MEF | λ –/– | 8-oxo-G | 8.9 |
| | λ –/– | Normal G | 0.1 |
| MEF | βλ –/– | 8-oxo-G | 8.2 |
| | βλ –/– | Normal G | 0.3 |
| MEF | + wt λ | 8-oxo-G | 9.2 |
| | + wt λ | Normal G | 0.1 |
| MEF | + λ T553A | 8-oxo-G | 9.4 |
| | + λ T553A | Normal G | 0.1 |

## Repair of 8-oxo-G:A mismatch in vivo by MutYH

As no tremendous differences could be detected between any of the transfection experiments with whatever Pol background, the suspicion emerged that such a gapped plasmid is been taken care of by any Pol present just at the time of transfection, and that the real importance is lying in the incorporation step *following* the action of MutYH. Thus, there were doubts that the gapped plasmid really reflects the real 8-oxo-G:A repair pathway, which, as mentioned, is thought to be initiated by the action of MutYH. It might thus be of importance that MutYH acts on the 8-oxo-G:A mismatch to recruit whatever repair Pol might be involved into the following 8-oxo-G bypass step. To address this question, plasmids containing a fully double-stranded insert with an 8-oxo-G:A mismatched base pair were transfected into mammalian cells and harvested at different time-points. The first goal was to determine the kinetics of the glycosylase activity of MutYH in such an experimental set-up *in vivo*, as had been shown already by Le Page *et al.* (141). In that paper the kinetics of MutYH activity was shown to be rather slow, yielding in Cos-7 and MRC5V1 cells only 35% of repair 12h after transfection and taking as long as 72h to observe complete repair of the 8-oxo-G:A mismatch.

When doing the experiments with transfection of the 8-oxo-G:A mismatch carrying plasmid for incubations times up to 96h, no repair could be observed by the AS-PCR approach in HeLa, Cos-1, 293T or MEF. This led inevitably to the question whether there was something wrong with the plasmid, or if the approach was just not correct. However, a personal communication with Eugenia Dogliotti (Rome) revealed, that obviously the data from the Le Page paper

couldn't be reproduced in her lab either, even when they were in possession of the exact plasmid that was used in the original publication.

Surprisingly, when the some of the extracts from those time-courses were transformed into bacteria and analysed via restriction digestion, a tendency towards increasing amounts of correct CG base pair establishment could be observed. This would indicate that some MutYH-initiated repair is taking place after all in the mammalian cells, but the AS-PCR somehow failed to detect this repair. Whether one of those two observations really holds true remains to be determined in future experiments.

Another issue that arose was the purity of the 8-oxo-G:A fully double-stranded plasmid. Analysis of preparations of these plasmids reveals a contamination of linear and/or nicked forms of the plasmid being present. These plasmids yield, after directly transforming them into *E.coli muty-/-* without prior passage through mammalian cells 81% of AT transformed progeny plasmids. This was surprising, as it has been shown various times that 100% of 8-oxo-G:A containing plasmids transformed into those bacteria yield approximately 64% of AT bearing progeny molecules. Therefore there was a fear, that the slight impurities in the plasmid preparations were influencing the result for some so far unclear reason. Due to this, an approach to produce 8-oxo-G:A containing plasmids via an alternative protocol modified after the approach by R. Woodgate's group (142) could be tested in future experiments.

## Final remarks for alternative approaches

As final remark, there are several lines of action that could be pursued in order to bypass the difficulties regarding the MutYH-related glycosylase activity.

One possibility is to try and use the 8-oxo-G:A plasmid in an *in vitro* system with mammalian whole cell extracts. This might enable the *in vitro* related difficulties to be overcome and could yield interesting data. However, the main focus – to demonstrate what is happening *in vivo* with 8-oxo-G bypass – would then be lost.

Another feasible option would be to construct a 8-oxo-G:A insert containing plasmid that harbours a mammalian origin of replication (in contrast to the pSKSL used so far, which does not allow for mammalian replication), as the activity of MutYH is thought to be closely linked to mammalian replication. For this end, a plasmid containing an oriP origin of replication (derived from the Epstein-Barr-Virus origin of replication) would need to be constructed. The oriP seems ideal for such a task, as it allows only for one replication per S phase in the mammalian cell, in contrast to many other viral origins that fire constantly and thus allow the lesion-bearing plasmid to 'escape' from detection by extensive replication.

# Summary of the Major Findings

In order to give a clear overview of the different important results obtained in the course of work on this thesis, a list of the major findings is given below.

- Pol λ is ubiquitinated by the E3 ligase CHIP *in vitro*.
- Pol λ is ubiquitinated by the E3 ligase Mule *in vitro* and *in vivo*.
- Ubiqutination of Pol λ by Mule influences cellular protein levels of Pol λ by targeting it for proteasomal degradation.
- Regulation of cellular levels of of Pol λ by Mule influences the capacity of mammalian cells to cope with 8-oxo-G lesions.
- Phosphorylation of Pol λ by Cdk2/CyclinA stabilizes protein levels of Pol λ by increasing its chromatin-association
- Increased chromatin-association of phosphorylated Pol λ is brought about by enhancing the affinity of Pol λ to chromatin-bound MutYH.
- Increased chromatin-binding of phosphorylated Pol λ counteracts the degradation of Pol λ by Mule.
- Pol ε, Pol β and Pol λ cooperate to bypass abasic sites residing in short DNA gaps.

# General Discussion

Right after hydrogen and helium, oxygen (O2) is by mass the third most abundant element occurring in the universe and the most abundant in our biosphere, air, sea and land (143). O2 is essential for the cellular respiratory chain in all aerobic organisms and is used in mitochondria, the cellular power plants, to help generate adenosine triphosphate (ATP) in a process called oxidative phosphorylation. ATP – sometimes referred to as 'molecular currency' of intracellular energy transfer - is the universal form of energy transport that is used by the cellular metabolism. The generation of ATP by the use of O2 is an extremely efficient and profitable process that is the basis of all vertebrate life on earth. However, as to everything else in life, besides the positive aspects there are also negative facets that originate from the use of O2 as a main source of energy. A main problem of O2 is its high reactivity that results in the formation of an immense array of oxidation products. Among those, oxidative DNA damage brought about by ROS is a dangerous event frequently occurring in any living organism. DNA damage caused by ROS can give rise to mutations, possibly result in cellular transformation and eventually give rise to cancer. In an attempt to combat the deleterious effects of oxidation damage in a cell, nature has devised a vast array of different repair mechanisms. In the review 'oxygen as a friend and enemy – how to combat the mutational potential of 8-oxo-guanine' (see introduction, (8)) the actors involved in BER of 8-oxo-G DNA damage were summarized. The mechanisms described outline the key proteins that might be important to make sure that the steady state level of oxidative damage remains low enough not to cause harm to the organism. Furthermore, in order to provide an update on the subject, the latest developments in this field are discussed following the manuscript.

As the basic ideas and mechanisms about the repair of oxidative DNA damage are beginning to unveil themselves, it is realized that the scientific community is still at an early stage of understanding the complete pathways and their regulation in detail. So far, most components of the BER pathway have been shown to be posttranslationally modified by phopshorylation, acetylation, methylation, ubiquitination and SUMOylation (139). However, the exact function of many of those modifications is currently a matter of speculation and we are still far away from understanding the intricate mechanisms that are in place to regulate and orchestrate the multitude of proteins that are involved in repair of oxidative DNA damage. More and more the general idea begins to emerge, that these PTMs could be the basis of regulation of the repair actors in time and space (133). A tight regulation of those proteins seems to be of particularly high importance as there is increasing evidence hinting towards the fact that de-regulation of Pol λ and also other TLS Pols including Pol β can lead to diseases in general (144) and to cancer in particular (136, 145).

The aim of this thesis was to shed more light on the intricate control mechanisms that are in place to regulate Pol λ's protein levels, its subcellular localization and its engagement into active repair complexes and thus to gain more insight into the regulation of Pol λ in vivo. The results of these studies can be found in the manuscript 'Regulation of oxidative DNA damage repair by DNA Polymerase λ and MutYH by cross-talk of phosphorylation and ubiquitination' that is attached.

Here, we identified Mule as an E3 ligase responsible for ubiquitination of Pol λ in vitro and in vivo, leading to degradation of Pol λ via the ubiquitin-proteasome pathway. Although Mule mainly monoubiquitinates Pol λ, the formation of di- or polyubiquitin chains could be observed as well. So far it remained unclear, whether Mule alone is responsible for the degradation of Pol λ, or if, like for Pol β (132), monoubiquitination by Mule is a signal to promote polyubiquitination by another E3 ligase. It remaims to be assessed, what possible role CHIP plays in the regulation of Pol λ levels in vivo, as it was also found to ubiquitinate pol λ in vitro (146). The investigation of CHIP's influence on the levels of Pol λ was initiated, but due to very contradictory results with the siRNA used to knock down CHIP, this line of analysis was postponed and is pending further investigation.

The regulation of Pol λ levels by Mule could be shown to influence the capacity of HEK 293T cells to perform correct bypass of 8-oxo-G, as assayed by single-nucleotide incorporation assays using crude cell extracts with siRNA-mediated knockdown of Mule compared to control cells. Importantly, it could be demonstrated that this repair is mainly carried out by Pol λ, as was shown with single-nucleotide incorporation experiments using Pol λ+/+ or Pol λ-/- MEF cell extracts depleted of Mule. Phosphorylation of Pol λ has previously been found to stabilize Pol λ by decreasing its ubiquitination (138). Fractionation experiments comparing HEK 293T cells stably transfected with Pol λ wt or the phosphorylation-defective Pol λ 4A mutant revealed a decrease in chromatin-association of Pol λ 4A. Thus, the phosphorylation-dependent chromatin binding protects and stabilizes Pol λ levels, as it prevents Pol λ from being shuttled to the cytoplasm. In the cytoplasm Pol λ gets ubiquitinated by Mule, which is a cytoplasmic protein, and is

subsequently degraded by the proteasome. Therefore, levels of Pol λ are controlled by means of changes in subcellular localization, which are dependent on the protein's phosphorylation status. This model is further corroborated by the fact that phosphorylation failed to show any influence on the amount of ubiquitination by Mule in vitro, excluding a direct effect of phosphorylation on ubiquitination of Pol λ by Mule (data not shown).

MutYH is the DNA glycosylase that catalyzes the excision of an incorrect A opposite 8-oxo-G. Its activity is followed by the action of Ape1 tailoring the gap with its 3' phosphodiesterase activity to accommodate a new base that will be incorporated by a Pol. Thus, MutYH precedes the role of Pol λ in incorporating a correct C opposite 8-oxo-G. It could be demonstrated that phosphorylation of Pol λ enhances its binding to MutYH and that depletion of MutYH in HEK 293T cells leads to a decrease in total cellular levels of Pol λ, as well as in the chromatin-associated Pol λ fraction. Phosphorylation of Pol λ by Cdk2/CyclinA occurs in the late S and G2 phase of the cell cycle. Considering that the replicative Pols δ and ε frequently misincorporate A opposite 8-oxo-G, high levels of A:8-oxo-G mispairs are expected to be present immediately after DNA synthesis in the S phase. Those mispairs have to be corrected before mitosis proceeds, as otherwise GC to TA transversion mutations can manifest themselves. In such a scenario, the Cdk2/CyclinA phosphorylation-dependent recruitment of Pol λ to chromatin makes a lot of sense, since Pol λ is so far the most likely candidate to work together with MutYH to achieve a correct repair of A:8-oxo-G lesions.

Unfortunately, the phosphorylation status of Pol λ in vivo could not be assessed due to the lack of suitable detection methods of phosphorylated Pol λ. Any attempt to generate phospho-specific antibodies for the detection of Pol λ in vivo has not been successful so far. Also, a change in mobility on conventional SDS-PAGE gels in endogenous Pol λ cannot be observed. The same problems apply to the detection of ubiquitinated endogenous Pol λ protein. Similarly, Pol β protein levels have recently shown to be regulated by Mule and CHIP in vivo (132, 134). Importantly, ubiquitin-mediated proteasomal degradation mainly regulates the protein levels of Pol β and not its activity.

So far there is no direct evidence indicating that ubiquitination of either Pol λ or β influences the choice of a specific Pol in the BER pathway directly. Rather, it seems that this Pol choice is brought about by the action of other PTMs, such as phosphorylation, enabling a subtle regulation of the subcellular localization. By regulating the subcellular localization, it is well possible that PTMs can contribute to the regulation of the degradation of a specific Pol, as is the case for Pol λ. It remains to be investigated whether the polymerase activity per se of any of the repair Pols can be stimulated directly by PTMs, if PTMs control the association with other proteins to enhance the formation entire repair complexes, or if their repair activity can be enhanced simply by changes in subcellular localization. Along this line, assessment of the impact of PTMs on the catalytic activity of Pol λ in vitro showed a slight inhibitory effect of ubiquitination (146) and failed to show any effect of phosphorylation on polymerization (data not shown).

The data presented in this thesis and the attached manuscripts, specifically only addressed the fine-tuning of Pol λ levels during physiological cellular conditions. The question regarding what happens upon induction of oxidative stress with Pol λ and its PTMs is still unsolved and elaborating further on this would be pure speculation. However, the regulatory mechanism that was identified in this work is important especially in the late S-and G2-phases of the cell cycle for the following line of argumentation. ROS encountering a C:G basepair during any cell cycle phase devoid of DNA synthesis will lead to the formation of a C:8-oxo-G base pair, a substrate for Ogg1 (reviewed in (8)). Subsequently, Ogg1 will remove the 8-oxo-G, and subsequent BER activity will take care of the residual AP site. Consequently, 8-oxo-G lesions caused by ROS in any of the non-replicative phases will mainly necessitate the action of Ogg1. A:8-oxo-G mispairs, however, are thought to arise from inaccurate bypass of an 8-oxo-G lesion by replicative Pols during the S phase. Therefore, late S and G2 are the phases during which the removal of A opposite 8-oxo-G is mainly needed. This is supported by the fact that protein levels of MutYH reach their maximum during S phase (147). Also, by means of shuttle-vector based studies, the repair of A:8-oxo-G mismatches in vivo by MutYH has been shown to be fourteen-fold more efficient when the substrate that is used is replication-proficient compared to a non-replicating one (148). These findings are in accordance with a replication-associated activity of MutYH. Thus, bearing in mind that Pol λ is stabilized by phosphorylation by Cdk2/CyclinA in late S and G2 as well (138), the stabilization of the interaction between MutYH and Pol λ during exactly these phases of the cell cycle might promote the accurate repair of A:8-oxo-G mismatches.

In conclusion, these results have shed a tiny bit of light onto the highly complex mechanisms that are in place to regulate the repair of oxidative DNA damage in time and space. Still, a more thorough understanding of the exact mechanisms that help cells to cope with oxidative DNA damage, and specifically their coordination and regulation in order to ensure their proper spatial and temporal distribution, is needed. More understanding regarding those aspects of DNA repair that are only at the beginning of being thoroughly addressed should lead to new insights as to what is going wrong when malignancies arise and, more importantly still, how to best combat them.

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# Curriculum vitae

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**Personal information**

- Date of Birth: 09.02.1983
- Place of birth: Zurich
- Nationality: CH and FIN

**Education**:

- 2005 – 2007: Promotion to **Dr. med. vet.,** University of Zurich
- 2002 – 2007: **Master in Veterinary medicine**, University of Zurich
- 1995 – 2002: High school, Kantonsschule Wiedikon, Zurich

**Work experience:**

- **02.2008 – 12.2011: University of Zurich, MD-PhD student**
  - Initiation, planning, execution and evaluation of own research projects
  - Presentation of scientific results in front of international scientific audience
  - Writing and publication of scientific articles
  - Compilation of intermediate and final project reports
  - Acquisition and management of project funding
  - Coordination and supervision of co-workers and research assistants
  - Advanced training in molecular biology and clinical research topics

- **09.2005 – 10.2007: ETH Zurich, Tutor for students of animal sciences**
  - Preparation of teaching contents for courses in anatomy and physiology
  - Theoretical and practical class-room training for students

- **08.2006 – 10.2006: Tierspital Zurich, Department of Veterinary Neurology**
  - Clinical training in Neurology
  - Communication of therapy plans and aftercare to patient owners

- **01.2005 – 12.2005: University of Zurich, Development of e-learning programs**
  - Responsible for establishment of ophthalmologic and surgical e-learning cases
  - Testing, implementing and presentation of software

- **09.2004 – 10.2005: Tierspital Zurich, Emergency anaesthetist**
  - Anesthetisation and monitoring of surgical patients pre-, intra- and postoperatively during nights and on weekends

- **02.2002 – 09.2002: City Hospital Triemli Zurich, Nurse aid**
  - Responsible for patient care under supervision

- **08.1997 – 10.2004: Equine sports stable Flond GR, Part-time stable manager**
  - Organizing and managing maintenance, training and healthcare of 12 horses
  - Giving riding lessons to children, adults and handicapped people
  - Assisting in the organization of equine sports events

- **2001 – 2003: Kantonsschulsportclub Wiedikon, Volleyball Trainer**
  - Co-manager of a junior Volleyball team

**Languages:**

- Finnish (mother tongue)
- German (first language)
- English (working language)
- French (fluent)
- Italian (fluent)

**Prizes and grants:**

- 2009: Swiss National Science Foundation, MD-PhD grant (155'000.- CHF)
- 2008: University of Zurich, Annual prize for best Dr. med. vet. Thesis
- 2007: University of Zurich, Grant for the MD-PhD project (97'812.- CHF)
- 2007: University of Zurich, best Veterinary medical student of the year

**Extracurricular activities:**

- Since 2009: First secretary of the Swiss MD-PhD association (SMPA)
- 2008- 2010: President of the organising committee for MD-PhD retreat
- 2009: Organiser of the symposium „Immunology–an update on hot topics"
- 2005 – 2006: President 'Veterinary students association', Vetsuisse Faculty Zurich
- 2004 – 2005: Vice president for organising of events, Vetsuisse Faculty, Zurich

**Interests and hobbies:**

- Preparation and training of young horses for participation in equine tournaments
- Golf
- Junior member of the Zurich Young Volleyball player's selection (SAR)

**Publication list:**


1. Smirnova E, Toueille M, **Markkanen E,** & Hubscher U (2005) The human checkpoint sensor and alternative DNA clamp Rad9-Rad1-Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery. Biochem J 389(Pt 1):13-17.

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6. **Markkanen E**, van Loon B, Ferrari E, Parsons JL, Dianov GL & Hubscher U (2012) Regulation of oxidative DNA damage repair by DNA polymerase lambda and MutYH by cross-talk of phosphorylation and ubiquitination. Proc Natl Acad Sci U S A 2012, 109, 437.

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